IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Prior Application: Examiner: K. Riskin Art Unit: 3312	I hereby certify that this 37 CFR 1.60 request and the documents referred to as attached therein are being deposited with the United States Postal Service on *MAY 5, 1998* in an envelope as "Express Mail Post Office to Addressee" service under 37 CFR 1.10, Mailing Label Number *EO239732 79 US* addressed to the: Assistant Commissioner for Patents, Washington, D.C. 20231 Casindra Himmelstein name of person signing *Casindra Himmelstein* signature

BOX PATENT APPLICATION
Assistant Commissioner for Patents
Washington, D.C. 20231

TRANSMITTAL OF FILING UNDER 37 CFR 1.60(b)

1.   This is a request for filing a

[X]   Divisional

[ ]   Continuation

application under 37 CFR 1.60 of pending prior application Serial No. 08/741,370 filed

on October 30, 1996 of Eric A. Wachter, Walter G. Fisher and H. Craig Dees for

Method For Improved Selectivity In Photo-Activation And Detection Of Molecular

Diagnostic Agents.

2.   True Copy of Original Prior Application Papers Attached.

I hereby verify that the attached papers are a true copy of the above-identified prior application, including the oath or declaration originally filed (37 CFR 1.60).

3.   Amendments

[ ]   Cancel in this application original claims _____ of the prior application before calculating the filing fee. (At least one original independent claim must be retained for filing purposes.)

[X]   Please enter the enclosed Preliminary Amendment (prior to calculating the filing fee)

4.   Information Disclosure Statement

[ ]   An Information Disclosure Statement is enclosed herewith.

5.   Fee Calculation (claims pending after entry of any amendments above)

[X]   A verified statement of small entity status:

[ ]   has been filed in the parent application and such status is still proper and desired

[X]   is attached

[ ]   Other than small entity

For	Number Filed	Number Extra	Rate sm/lg ent.	Fee
Total Claims	10	-20 = 0	x $11/22	$0
Independent Claims	2	- 3 = 0	x $41/82	$0
Multiple Dependent Claims	No		$270/135	$0
Basic Fee (minimum amount required)			$395/790	$395
Assignment			$40	$0
Total Filing Fee				$395

6. Fee Payment

[X] A check in the amount of $ 395.00 is enclosed

[ ] Please charge deposit account 03-3135 in the amount of ____.

[X] The Commissioner is hereby authorized to charge any additional fees which may be required, or credit any overpayment to Account No. 03-3135. A duplicate copy of this sheet is enclosed.

7. Drawings

[ ] New formal drawings are enclosed.

[X] Informal drawings are enclosed.

8. Foreign Priority

[ ] Priority of PCT application serial no. _ filed on _ is claimed under 35 U.S.C. 119.

[ ] The certified copies has been filed in prior U.S. application Serial No. _____.

[ ] The certified copy will follow.

9.   Relate Back -- 35 U.S.C. 120

[X]   Please amend the specification by inserting before the first line the sentence:

--This application is a _divisional_ of copending U.S. application Serial No.

08/741,370, filed on _October 30, 1996.--

10.   Inventorship

With respect to the prior copending U.S. application from which this application claims benefit under 35 USC 120 the inventor(s) in this application is (are):

[X]   the same

[ ]   less than those named in the prior application and it is requested that the following inventor(s) identified above for the prior application be deleted:

_____

11.   Assignment

[X]   The prior application is assigned of record to _Photogen, Inc.._

[ ]   An assignment of the invention to _____ is attached (with cover sheet) for recording..

12.   Maintenance of Copendency of Prior Application

[ ]   A petition, fee and response has been filed to extend the term in the pending prior application until ___. or

[ ]   A conditional petition for extension of time is being filed in the pending parent application (copy enclosed).

13. Power of Attorney

    [X]   The power of attorney in the prior application is to: Edward D. Manzo ,
            registration no.   28,139

    [ ]   The power appears in the original papers in the prior application.

    [X]   Since the power does not appear in the original papers, a copy of the
            power in the prior application is enclosed.

    [X]   Address all future communications to:

                Edward D. Manzo
                Cook, McFarron & Manzo, Ltd.
                200 W. Adams St., Ste. 2850
                Chicago, IL 60606

                Respectfully submitted,

Date: *May 5, 1998*
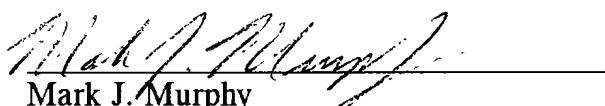
                Mark J. Murphy
                Registration no. 34,225

COOK, MCFARRON & MANZO, LTD.
200 W. Adams Street
Suite 2850
Chicago, Illinois 60606
(312) 236-8500

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of:)
)
    Eric Wachter et al.   )   "Express Mail" Mailing Label No. _EL0?347_ _327945_
)
)   Date of Deposit _May 5, 1998_
Serial No.:   )
)   I hereby certify that this paper or fee is being
Filed: Herewith   )   deposited with the United States Postal Service
)   "Express Mail Post Office to Addressee" service
For: Method For Improved Selectivity)   under 37 CFR 1.10 on the day indicated above
    In Photoactivation And   )   and is addressed to the Assistant Commissioner
    Detection Of Molecular   )   for Patents, Washington, D.C. 20201.
    Diagnostic Agents   )   Name _Casandra Himmelstein_
)   (typed or printed)
Examiner:)
)   Signature _Casandra Himmelstein_
Art Unit:)

    May 4, 1998

Assistant Commissioner for Patents
Washington, D.C. 20231

PRELIMINARY AMENDMENT A

Prior to calculation of the fees and examination, please enter the following amendment in the above-identified application as follows:

IN THE SPECIFICATION:

Please amend the specification as follows:

Page 11, lines 8 and 11, change "dectected" to --detected--.

IN THE CLAIMS

Please amend the claims as follows:

Cancel Claims 1-25.

Please add the following new claims:

--New Claim 26. Imaging apparatus for imaging a particular volume of plant or animal tissue containing at least one photo-active molecular agent, the apparatus comprising:

a source of collimated light, said light having a frequency effective to penetrate substantially into the tissue, said light being adapted to promote simultaneous two-photon excitation (TPE) of the molecular agent contained within the tissue;

focusing apparatus for focusing the collimated light throughout a range of focal lengths extending from a surface of said tissue to a depth substantially beyond said surface, said light source and focusing apparatus cooperating to promote TPE of the molecular agent,

wherein a focal point or focal plane is adjustable with respect to said tissue; and

a detector located proximate to the tissue and positioned to detect said light emitted by the molecular agent and which travels a path that does not retrace an optical path of the light incident on the tissue, said detector configured to produce a detected signal characteristic of the particular volume at which the light source has been focused.--

--New Claim 27. The apparatus of Claim 26 wherein said light source produces a pulsed output having a pulse repetition frequency above about 75 megahertz and a sub-nanosecond pulse duration.--

--New Claim 28. The apparatus of Claim 27 wherein said light source produces near-infrared light.--

2

--New Claim 29. The apparatus of Claim 28 wherein said light source produces pulse energies of about 20 nanojoules.--

--New Claim 30. The apparatus of Claim 28 wherein said light source comprises a laser.--

--New Claim 31. The apparatus of Claim 26 further comprising a processor coupled to said detector.--

--New Claim 32. The apparatus of Claim 31 further comprising a modulation system associated with said light source, said processor being coupled to said modulation system.--

--New Claim 33. Apparatus for medical diagnostic imaging comprising:

light source means for directing a confined light at and into deep tissue to be imaged, said light being selected in frequency and energy to penetrate below a tissue surface and to promote TPE substantially only at a confocal region;

means for varying a position of a confocal region of the light within a range of depths in the tissue to be imaged; and

detector means positioned to receive and detect isotropic radiation emitted by a photo-activated molecular agent within the tissue after said agent has been excited using two-photon excitation.--

--New Claim 34. The apparatus of Claim 33:

wherein the light source means includes means for producing a collimated light beam; and

wherein the light source means includes focusing means for focusing the collimated light beam to a confocal region located with tissue at a point below the tissue surface.--

--New Claim 35. The apparatus of Claim 34 wherein the means for producing a collimated light beam comprises a pulsed laser operating the near infra-red spectrum.--


REMARKS

This preliminary amendment is to add those claims which were added in the parent application in a preliminary amendment but were subject to a restriction requirement.

Favorable consideration is earnestly solicited. If any additional fee should be due, please charge our Deposit Account 03-3135.

<div align="right">Respectfully submitted,</div>

Dated: May 4, 1998
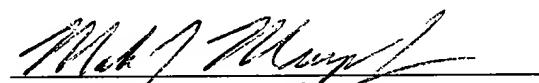
Mark J. Murphy
Registration no: 34,225

COOK, McFARRON & MANZO, LTD.
200 W. Adams Street
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PATENT APPLICATION

METHOD FOR IMPROVED SELECTIVITY IN PHOTO-ACTIVATION AND DETECTION OF MOLECULAR DIAGNOSTIC AGENTS

Inventors: Eric A. Wachter
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Oak Ridge, Tennessee 37830

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Knoxville, Tennessee 37923

EI0239

"Express Mail" Mailing Label No. 73279US
Date of Deposit MAY 5, 1998

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Name Casindra Himmelstein
(typed or printed)

Signature Casindra Hulst

Richard M. Kessler, Esq.
11324 Gates Mill Drive
Knoxville, Tennessee, 37922-1752
(423) 777-0244

Docket No. PHO-0002

* * *

"Express Mail" mailing label number   EG609572792US
Date of Deposit   October 30, 1996
    I hereby certify that this paper or fee is being deposited with the United States Postal Service "Express Mail Post Office to Addressee" service under 37 CFR 1.10 on the date indicated above and is addressed to: Commissioner of Patents and Trademarks, Washington, D.C. 20231, on this date.

    Richard M. Kessler
(Typed or printed name of person mailing paper or fee)

R.M. Kessler
(Signature of person mailing paper)

METHOD FOR IMPROVED SELECTIVITY IN PHOTO-ACTIVATION AND DETECTION OF MOLECULAR DIAGNOSTIC AGENTS

This invention was made with Government support under Contract No. DE-AC05-84OR21400 awarded by the U.S. Department of Energy to Lockheed Martin Energy Systems, Inc. Lockheed Martin Energy Systems and the Oak Ridge Associated Universities have waived rights to this invention to the inventors. The Government has rights in this invention pursuant to Contract

5 No. DE-AC05-84OR21400 awarded by the U.S. Department of Energy.

BACKGROUND OF THE INVENTION:

Field of the Invention:

The present invention relates generally to methods and apparatus for remotely effecting spatially-selective photo-activation of one or more molecular agents and for improving the detection

10 of the diagnostic signals thereby produced. The method taught for effecting photo-activation utilizes the special properties of non-linear optical excitation for promoting an agent from one molecular energy level to another with a high degree of spatial and molecular specificity. The special features of this method are applicable for activation of various endogenous and exogenous imaging agents, and in particular afford distinct advantages in the diagnosis of diseases in humans

15 and animals. Specifically, use of non-linear excitation methods facilitate controlled activation of diagnostic agents in deep tissue using near infrared to infrared radiation, which is absorbed and scattered to a lesser extent than methods and radiations currently used. Combination of these non-linear excitation methods with advanced signal encoding and processing methods greatly increases sensitivity in the detection of diagnostic signals.

20

Method for Improved Selectivity in Photo-Activation
and Detection of Molecular Diagnostic Agents
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Description of the Prior Art:

       An urgent need exists in many fields, and especially in the medical diagnostics field. for a method that is capable of selectively controlling the remote activation of various molecular agents while producing few if any side effects resulting from the activation process. The desired

5   improvements in activation include enhancements in spatial or temporal control over the location and depth of activation, reduction in undesirable activation of other co-located or proximal molecular agents or structures, and increased preference in the activation of desirable molecular agents over that of undesirable molecular agents. Various linear and non-linear optical methods have been developed to provide some such improvements for some such agents under very

10   specialized conditions. However, in general the performance and applicability of these methods have been less than desired. Specifically, improved photo-activation methods are needed that may be used to selectively photo-activate a variety of molecular diagnostic agents while providing improved performance in the control of application of this photo-activation.

       Application of optical radiation as a means for remotely activating molecular probes has

15   been known for many years. Specifically, linear optical excitation methods have been used extensively as a means for achieving semi-selective activation of molecular diagnostic agents. Linear optical excitation occurs when a target agent, such as a molecular diagnostic agent, undergoes a specific photo-chemical or photo-physical process, such as fluorescent emission, upon absorption of energy provided by a single photon. These processes can in many cases be

20   very efficient, and use of such processes is attractive for numerous applications. Unfortunately, performance of these linear methods have not always been as successful as desired. For example, there is strong evidence that ultraviolet radiation used to excite some molecular probes can produce

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disease in humans and animals, such as induced skin cancer, along with other undesirable side

effects. Furthermore, a less than desirable penetration depth has plagued most efforts at linear

optical excitation of molecular agents, primarily as a consequence of the effects of optical scatter

and of absorbance of the incident probe radiation at wavelengths near the linear absorption bands

5   of these agents. As an example, Wachter and Fisher (E.A. Wachter and W.G. Fisher, "Method

and Apparatus for Evaluating Structural Weakness in Polymer Matrix Composites." U.S. Patent

5,483,338) teach of a rapid optical method capable of sensitively imaging chemical transformations

in probe molecular agents; however, due to scatter and absorbance of the incident probe radiation,

the method is only suitable for topical analysis. Vo-Dinh and co-workers (T. Vo-Dinh, M.

10   Panjehpour, B.F. Overholt, C. Farris, F.P. Buckley III and R. Sneed, "In-Vivo Cancer-Diagnosis

of the Esophagus Using Differential Normalized Fluorescence (Dnf) Indexes," Lasers in Surgery

and Medicine, 16 (1995) 41-47; and M. Panjehpour, B.F. Overholt, J.L. Schmidhammer, C.

Farris, P.F. Buckley, and T. Vo-Dinh, "Spectroscopic Diagnosis of Esophageal Cancer: New

Classification Model, Improved Measurement System," Gastrointestinal Endoscopy, 41 (1995)

15   577-581) teach of the use of similar linear optical probe methods for detection of diseased tissues

in humans; however, this approach is also plagued by less than desirable penetration depth and is

limited to detection of superficial lesions due to scatter and absorption of the incident probe

radiation. Also, because this type of excitation is linearly related to excitation power, such

methods provide no effective means for limiting the location of probe excitation along the optical

20   path. In fact, virtually all examples of the use of linear optical excitation are plagued by

fundamental performance limits that are attributable to undesirable absorption and scatter of the

incident optical radiation by the surrounding matrix, poor specificity in excitation of probe

Method for Improved Selectivity in Photo-Activation
and Detection of Molecular Diagnostic Agents
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molecular species, and a lack of suitable physical mechanisms for precise control of the extent and depth of activation.

Various non-linear optical excitation methods have been employed in an effort to achieve specific improvements in the selectivity of photo-activation for certain applications, and to address

5   many of the limitations posed by linear excitation methods. In fact, the non-linear process consisting of simultaneous absorption of two photons of light by a molecule to effect excitation equivalent to that resulting from absorption of a single photon having twice the energy of these two photons is very well known, as are the specific advantages of this process in terms of reduced absorption and scatter of excitation photons by the matrix, enhanced spatial control over the region

10   of excitation, and reduced potential for photo-chemical and photo-physical damage to the sample. Excitation sources ranging from single-mode, continuous wave (CW) lasers to pulsed Q-switched lasers having peak powers in excess of 1 GW have been employed for numerous examples of two-photon excitation methods. For example, Wirth and Lytle (M.J. Wirth and F.E. Lytle, "Two-Photon Excited Molecular Fluorescence in Optically Dense Media," Analytical Chemistry, 49

15   (1977) 2054-2057) teach use of non-linear optical excitation as a means for stimulating target molecules present in optically dense media; this method is shown to be useful in limiting undesirable direct interaction of the probe radiation with the media itself, and provides a means for effectively exciting target molecular agents present in strongly absorbing or scattering matrices. Improved spatial control over the active region has been further developed by Wirth (M.J. Wirth

20   and H.O. Fatunmbi, "Very High Detectability in Two-Photon Spectroscopy," Analytical Chemistry, 62 (1990) 973-976); specifically, Wirth teaches a method for achieving extremely high spatial selectivity in the excitation of target molecular agents using a microscopic imaging system.

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Similar control has been further applied by Denk et al. (W. Denk, J.P. Strickler and W.W. Webb, "Two-Photon Laser Microscopy," U.S. Patent No. 5,034,613) who teach of a special epi-illumination confocal laser scanning microscope utilizing non-linear laser excitation to achieve intrinsically high three-dimensional control in the photo-activation of various molecular fluorophor

5   agents on a cellular or sub-cellular scale. This microscope is used to excite molecular fluorophor agents added to biological specimens, which constitute an optically dense medium; the special properties of non-linear two-photon excitation are utilized to substantially limit excitation and subsequent detection of the fluorescent signal thus produced to a confocal region occurring at the focus of an objective lens, thereby enhancing contrast in three-dimensional imaging by sharply

10   controlling the depth of focus. Emitted fluorescent light is collected by the excitation objective using an epi-illumination configuration. Control of photo-excitation for generation of luminescence-based images at the cellular and sub-cellular level is shown in target samples mounted on a stage. Furthermore, Denk teaches that reduction in photo-induced necrosis of cells located at the focal plane is a primary benefit of this microscopy approach, based on the

15   replacement of ultraviolet excitation radiation with less damaging near infrared excitation radiation.

In later work by Denk et al. (W. Denk. D.W. Piston and W.W. Webb, "Two-Photon Molecular Excitation in Laser-Scanning Microscopy," in Handbook of Biological Confocal Microscopy, Second Edition, J.B. Pawley. ed., Plenum Press, New York, 1995, pp. 445-458) an external whole area detection method is taught for collection of microscopic imaging data produced

20   from two-photon excited fluorescent tags. This method, which the authors state as being "as yet untried," eliminates the need to collect backscattered fluorescent light using epi-illumination (see p. 452). Denk points out that this approach could be useful if the microscope objective does not

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transmit the emitted fluorescent wavelengths, but that it is "vulnerable to contamination from ambient room light." In this work and in the earlier Denk patent (5,034,613), no apparent method is used or anticipated for reduction of background interference from either ambient light or from scattered excitation light.

5   In fact, the well known low efficiency of the two-photon excitation process can translate into a very high ratio of scattered. unabsorbed excitation light to fluorescence emission. Use of various modulation methods for reduction of interference from scattered excitation light, as well as from interferences from ambient light and from other environmental and instrumental background sources, has numerous precedents. In the field of two-photon excited fluorescence, Lytle and co-

10   workers (R.G. Freeman, D.L. Gilliland and F.E. Lytle, "Second Harmonic Detection of Sinusoidally Modulated Two-Photon Excited Fluorescence," Analytical Chemistry, 62 (1990) 2216-2219; and W.G. Fisher and F.E. Lytle, "Second Harmonic Detection of Spatially Filtered Two-Photon Excited Fluorescence," Analytical Chemistry, 65 (1993) 631-635) teach sophisticated methods for rejection of scattered laser excitation light by making use of second-harmonic detection

15   methods: when sinusoidal modulation of the excitation light is performed at one frequency, and detection of the two-photon excited fluorescence is performed at twice that frequency (which is the second harmonic of the excitation modulation frequency), interferences from scattered excitation light are virtually eliminated. And by proper selection of the modulation frequency to avoid electronic and other noise frequencies, rejection of instrumental and environmental interferences is

20   extremely high.

       Hence, it is well known that two-photon excitation of fluorescence can be used under laboratory conditions to excite molecular fluorophors using light at approximately twice the

Method for Improved Selectivity in Photo-Activation
and Detection of Molecular Diagnostic Agents
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wavelength of that used for linear single-photon excitation, and that the excitation thereby effected can improve three-dimensional spatial control over the location of excitation, can reduce interference from absorption and scatter of the excitation light in optically dense media, and can reduce collateral damage along the excitation path to living cell samples undergoing microscopic

5   examination.

Nonetheless, while the substantial body of prior art exemplified by these cited examples clearly demonstrates many attractive features of various photo-activation methods that are applicable for diagnostic and other in vivo microscopic imaging uses, a general method for achieving selective photo-activation of one or more molecular agents with a high degree of spatial

10   control that is capable of meeting the diverse needs of the medical diagnostic industry has not been previously taught. Specifically, practical methods for effecting such control on scales that are significant for medical diagnostic applications have not been previously taught.

It is, therefore, an object of the present invention to provide a general method for achieving selective photo-activation of one or more molecular agents with a high degree of spatial control.

15   It is another object of the present invention to provide such a method that is capable of meeting the diverse needs of the medical diagnostic industry.

It is another object of the present invention to provide a practical method for effecting such control on scales that are significant for medical diagnostic applications.

SUMMARY OF THE INVENTION:

20   Having regard to the above and other objects and advantages, the present invention generally provides for a method for the imaging of a particular volume of plant or animal tissue, wherein the plant or animal tissue contains at least one photo-active molecular agent. The method

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comprises the steps of treating the particular volume of the plant or animal tissue with light

sufficient to promote a simultaneous two-photon excitation of the photo-active molecular agent

contained in the particular volume of the plant or animal tissue, photo-activating at least one of the

at least one photo-active molecular agent in the particular volume of the plant or animal tissue,

5   thereby producing at least one photo-activated molecular agent, wherein the at least one photo-

activated molecular agent emits energy, detecting the energy emitted by the at least one photo-

activated molecular agent, and producing a detected energy signal which is characteristic of the

particular volume of plant or animal tissue. The present invention also provides a method for the

imaging of a particular volume of material, wherein the material contains at least one photo-active

10   molecular agent.

In a preferred embodiment of the present invention, the light sufficient to promote a

simultaneous two-photon excitation of the at least one photo-active molecular agent is laser light. It

is also preferred that the light sufficient to promote a simultaneous two-photon excitation of the

photo-active molecular agent is a focused beam of light, and more preferred that the focused beam

15   of light is focused laser light.

Another preferred embodiment of the present invention further includes a first step of

treating the material, plant tissue or animal tissue with at least one photo-active molecular agent,

wherein the particular volume of the material, plant tissue or animal tissue retains at least a portion

of the at least one photo-active molecular agent. It is more preferred that the at least one photo-

20   active molecular agent is selected from the group consisting of psoralen, 5-methoxypsoralen (5-

MOP), 8-methoxypsoralen (8-MOP), 4,5',8-trimethylpsoralen (TMP), 4'-aminomethyl-4,5',8-

trimethylpsoralen (AMT), 5-chloromethyl-8-methoxypsoralen (HMT), angelicin (isopsoralen), 5-

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methylangelicin (5-MIP), 3-carboxypsoralen, porphyrin, haematoporphyrin derivative (HPD),

photofrin II, benzoporphyrin derivative (BPD), protoporphyrin IX (PpIX), dye haematoporphyrin

ether (DHE), polyhaematoporphyrin esters (PHE), 13,17-N,N,N-dimethylethylethanolamine ester

of protoporphyrin (PH1008), tetra(3-hydroxyphenyl)-porphyrin (3-THPP), tetraphenylporphyrin

monosulfonate (TPPS1), tetraphenylporphyrin disulfonate (TPPS2a), dihaematoporphyrin ether.

mesotetraphenylporphyrin, mesotetra(4N-methylpyridyl)porphyrin (T4MpyP), octa-(4-*tert*-

butylphenyl)tetrapyrazinoporphyrazine (OPTP), phthalocyanine, tetra-(4-*tert*-butyl)phthalocyanine

(t_4-PcH$_2$), tetra-(4-*tert*-butyl)phthalocyanatomagnesium (t_4-PcMg), chloroaluminum sulfonated

phthalocyanine (CASPc), chloroaluminum phthalocyanine tetrasulfate (AlPcTS), mono-sulfonated

aluminum phthalocyanine (AlSPc), di-sulfonated aluminum phthalocyanine (AlS2Pc), tri-

sulfonated aluminum phthalocyanine (AlS3Pc), tetra-sulfonated aluminum phthalocyanine

(AlS4Pc), silicon phthalocyanine (SiPc IV), zinc II phthalocyanine (ZnPc), bis(di-isobutyl

octadecylsiloxy)silicon 2,3-naphthalocyanine (isoBOSINC), germanium IV octabutoxyphthalo-

cyanine (GePc), rhodamine 101 (Rh-101), rhodamine 110 (Rh-110), rhodamine 123 (Rh-123),

rhodamine 19 (Rh-19), rhodamine 560 (Rh-560), rhodamine 575 (Rh-575), rhodamine 590 (Rh-

590), rhodamine 610 (Rh-610), rhodamine 640 (Rh-640), rhodamine 6G (Rh-6G), rhodamine

700 (Rh-700), rhodamine 800 (Rh-800), rhodamine B (Rh-B), sulforhodamine 101, sulfo-

rhodamine 640, sulforhodamine B, coumarin 1, coumarin 2, coumarin 4, coumarin 6, coumarin

6H, coumarin 7, coumarin 30, coumarin 47, coumarin 102, coumarin 106, coumarin 120,

coumarin 151, coumarin 152, coumarin 152A, coumarin 153, coumarin 311, coumarin 307,

coumarin 314, coumarin 334, coumarin 337, coumarin 343, coumarin 440, coumarin 450,

coumarin 456, coumarin 460, coumarin 461, coumarin 466, coumarin 478, coumarin 480,

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coumarin 481, coumarin 485. coumarin 490, coumarin 500, coumarin 503, coumarin 504.

coumarin 510, coumarin 515. coumarin 519, coumarin 521, coumarin 522, coumarin 523,

coumarin 535, coumarin 540, coumarin 540A, coumarin 548, 5-ethylamino-9-diethylamino-

benzo[a]phenoxazinium (EtNBA), 5-ethyl-amino-9-diethyl-aminobenzo[a]phenothiazinium

5   (EtNBS), 5-ethylamino-9-diethylaminobenzo[a]pheno-selenazinium (EtNBSe), chlorpromazine,

chlorpromazine derivatives, chlorophyll derivatives, bacteriochlorophyll derivatives, metal-ligand

complexes, tris(2,2'-bipyridine)ruthenium (II) dichloride (RuBPY), tris(2,2'-bipyridine)rhodium

(II) dichloride (RhBPY), tris(2,2'-bipyridine)platinum (II) dichloride (PtBPY), pheophorbide a,

merocyanine 540, vitamin D, 5-amino-laevulinic acid, photosan, chlorin e6, chlorin e6 ethylene-

10   diamide, mono-L-aspartyl chlorin e6, and phenoxazine Nile blue derivatives, stilbene. stilbene

derivatives, and 4-(N-(2-hydroxyethyl)-N-methyl)-aminophenyl)-4'-(6-hydroxyhexylsulfonyl)-

stilbene (APSS). It is also more preferred that the at least one photo-active molecular agent is at

least one biogenic photo-active molecular agent that is specific to a particular material or tissue

within the particular volume of material, plant tissue or animal tissue, even more preferred that the

15   at least one biogenic photo-active molecular agent includes a segment selected from the group

consisting of DNA, RNA, amino acids, proteins, antibodies, ligands, haptens, carbohydrate

receptors or complexing agents, lipid receptors or complexing agents, protein receptors or

complexing agents, chelators. and encapsulating vehicles and yet further more preferred that the at

least one biogenic photo-active molecular agent further includes a segment which is photo-activated

20   when subject to light sufficient to promote a simultaneous two-photon excitation.

In yet another preferred embodiment of the present invention, the step of treating the

particular volume of the material, plant tissue or animal tissue with light sufficient to promote a

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PHO-0002   - Specification Page 10 of 62 -

simultaneous two-photon excitation of the at least one photo-active molecular agent contained in the

particular volume of the material, plant tissue or animal tissue further includes the step of

modulating light from a light source with a particular type of modulation , thereby producing a

modulated light, and the step of treating the particular volume of the material. plant tissue or animal

5   tissue with the modulated light sufficient to promote a simultaneous two-photon excitation of the at

least one photo-active molecular agent contained in the particular volume of the material, plant

tissue or animal tissue. It is also preferred that the present invention further include the steps of

demodulating the dectected energy signal with the particular type of modulation, and producing a

demodulated energy signal which is characteristic of the particular volume of the material, plant

10   tissue or animal tissue.

It is more preferred that the step of demodulating the dectected energy signal with the

particular type of modulation includes demodulating the detected energy signal at a frequency twice

that of the particular type of modulation, thereby detecting the second harmonic of the particular

type of modulation. It is also more preferred that the demodulated energy signal which is

15   characteristic of the particular volume of the material, plant tissue or animal tissue represents a

change in lifetime of at least one photo-activated molecular agent present in the particular volume of

the material, plant tissue or animal tissue.

BRIEF DESCRIPTION OF THE DRAWINGS:

The above and other features and advantages of the invention will become further known

20   from the following detailed description of preferred embodiments of the invention in conjunction

with the drawings in which:

FIGURE 1 shows example energy level diagrams for linear and non-linear optical

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excitation;

FIGURE 2 shows the relationships between incident power distribution and excitation efficiency for single-photon and two-photon excitation;

FIGURE 3 shows an example absorption spectrum for animal tissue covering the

5   ultraviolet to near infrared spectral region;

FIGURE 4 shows a scattering spectrum for animal tissue covering the ultraviolet to near infrared spectral region;

FIGURE 5 shows the general trends in optical absorption and scattering properties of tissue for incident short wavelength and long wavelength light;

10   FIGURE 6 compares optically-induced excitation regions in tissue when single-photon and two-photon excitation methods are used;

FIGURE 7 shows typical properties of linear excitation of a diagnostic agent in solution;

FIGURE 8 shows typical properties of non-linear excitation of a diagnostic agent in solution;

15   FIGURE 9 shows a photograph of two-photon excited fluorescence of the dye molecule coumarin 480 distributed evenly throughout a tissue phantom;

FIGURE 10 shows a photograph of two-photon excited fluorescence of the dye molecule coumarin 480 distributed evenly throughout a tumor specimen;

FIGURE 11 shows a diagram of a specific preferred embodiment of the subject invention

20   for imaging endogenous or exogenous diagnostic imaging agents;

FIGURE 12 shows a diagram of an alternate preferred embodiment of the subject invention for imaging endogenous or exogenous diagnostic imaging agents, wherein modulation is used to

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improve imaging performance; and

FIGURE 13 shows a diagram of a second alternate preferred embodiment of the subject invention for videographic imaging of superficial features.

DETAILED DESCRIPTION OF THE DRAWINGS:

5   The invention described here utilizes the unique physical properties of non-linear optical excitation of molecular agents to effect improved spatial control over the photo-activation of those agents. In addition, non-linear optical excitation is shown to have further advantages during photo-activation of medical diagnostic and other agents, including reduction of collateral excitation and damage along the excitation path, reduction in exposure to harmful optical wavelengths, and

10   reduction of interference from absorption and scattering processes originating from the environment surrounding the excited agent.

The fundamental significance of the invention taught in this disclosure lies in the use of non-linear, simultaneous two-photon optical excitation processes to remotely photo-activate one or more molecular diagnostic agent with a high degree of spatial control and improved depth of

15   penetration. These molecular agents may be exogenous agents added to the system under examination, or they may be endogenous components of the system. Example exogenous diagnostic agents include various psoralen derivatives, while example endogenous agents include aromatic amino acids and nucleic acids. Two-photon excitation is performed at a wavelength approximately twice that of corresponding single-photon absorbance bands. By focussing a beam

20   of optical radiation into a specimen under examination, the diagnostic agent may be excited at a location substantially limited to the confocal region of the focussed beam. The confocal region, Z_c, is defined as the zone extending a distance of $2\pi w_0^2 / \lambda$, where w_0 is the diameter of the

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minimum beam waist and λ is the wavelength of the optical radiation. In contrast, when linear excitation methods are employed, excitation occurs substantially along the entire optical path. making spatial localization of excitation considerably less defined. Thus, use of the two-photon excitation process greatly increases the resolution of excitation along the optical path. Further.

5   since excitation is performed at long wavelengths relative to corresponding linear excitation processes, scatter and absorption of the excitation energy is greatly reduced. For thick, optically dense samples, such as human tissue, this means that two-photon excitation is possible at depths considerably greater than is possible using linear excitation methods. It is not necessary for the light emitted from the diagnostic agent to be detected or imaged directly without scatter, since

10   spatial information concerning the origin of the emitted light is encoded by and may be correlated to the excitation focus. By moving the location of this focus relative to the specimen, a two- or three-dimensional image of the emitted light may be developed. Also, by modulating the excitation light and using an appropriate demodulation method on the detection apparatus, rejection of scattered excitation light and other interferences may be markedly improved.

15   The present invention is intended primarily for in vivo detection and imaging of disease and other characteristics of tissues, such as cancer in the human breast. However, it will be clear once the invention is fully disclosed that the methods and apparatus taught have numerous additional applications, and that these methods and apparatus can be applied to the field of two-photon laser scanning microscopy, as taught by Denk et al., to achieve substantive improvements in the

20   performance characteristics of such instruments. To begin this full disclosure, a review of the fundamental physics underlying linear and non-linear optical excitation will be useful.

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Comparison of linear and non-linear excitation – energy level diagram formulation:

FIGURE 1 shows typical molecular energy level diagrams for several linear and non-linear optical excitation processes. In this representation, which consists of simplified Jablonski diagrams, the vertical direction corresponds to a change in energy, while the horizontal direction represents the sequence of events, progressing from the left to right. Solid horizontal lines represent quantum mechanically allowed molecular energy levels, while dashed horizontal lines represent disallowed, virtual energy levels. Quantum mechanically allowed molecular energy levels are relatively long lived and the probability of excitation of a molecule upon absorption of energy, such as that provided by absorption of a photon of appropriate energy, is high. Virtual energy levels may be reached through a variety of excitation processes, but in contrast to allowed molecular transitions they have exceedingly short lifetimes (on the order of 10^{-15} s, as predicted by the Heisenberg uncertainty principle), making them significant only under special excitation conditions. Straight arrows in Jablonski diagrams represent radiative energy transfer processes: upward arrows indicate absorption of energy, while downward arrows represent radiative emission, such as fluorescent or phosphorescent emission of a photon. Crooked arrows represent non-radiative energy transfer processes, such as vibrational relaxation. The vertical length of the straight or crooked arrows is proportional to energy absorbed or emitted in a given process.

For the first Jablonski diagram shown in FIGURE 1, single-photon excitation to an allowed energy level 2 occurs upon absorption of a photon 4 having sufficient energy to directly promote the molecule from a first allowed electronic energy level 6 (generally the lowest electronic energy level, or ground state, denoted as S_0) to a second allowed electronic energy level 8 having a higher overall energy level (represented here as the S_1 state). Note that there may be multiple

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allowed higher electronic energy levels to which excitation may occur, and that these are typically

denoted S_1, S_2, and so on as their energy increases. The nomenclature S_1 indicates a singlet

electronic energy level that conforms to the Pauli exclusion principle, wherein the spins of all

electrons are paired and these paired electron spins are opposite to one another. One or more triplet

5   excited states 10 may also be possible for some molecular systems, with the example here denoted

as T_1. Triplet states differ from singlet states in that the spins of all electrons are paired except for

two. Each allowed electronic energy level (singlet or triplet) may be further subdivided into an

ensemble of discrete vibrational levels 12; each of these discrete vibrational levels 12 may in turn

be further subdivided into an ensemble of discrete rotational energy levels. Hence, each allowed

10 electronic energy level, S_0, S_1, T_1, and so on. constitutes a complex band of allowed energy levels

due to the large number of possible vibrational and rotational states possible. Upon absorption of

energy from a photon 4 the molecule is promoted to a particular unique electronic and vibrational

level 14, sometimes referred to as a vibronic level. From this excited state the molecule can then

undergo rapid internal conversion 16, for example to the lowest allowed excited vibronic energy

15 level 18 in the second allowed electronic energy level 8. This internal conversion 16 is typically

very fast, occurring on a time scale on the order of 10^{-12} to 10^{-15} sec. Finally, the excited

molecule can undergo further relaxation, such as through collisional deactivation 20, to return to

the initial, first energy level 6. Alternative relaxation processes include fluorescent emission of a

photon 21, which occurs directly from S_1 to S_0, and phosphorescence, which occur following

20 intersystem crossing 22 from a singlet state to a triplet state 10. Note that singlet to singlet

electronic transitions, such as those shown for $S_1 \rightarrow S_0$, constitute quantum mechanically allowed

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transitions according to the Pauli exclusion principle. In contrast, transitions from a singlet to a triplet state 10, such as $S_1 \rightarrow T_1$, are quantum mechanically forbidden since the electron spins do not remain paired. However, the probability of internal conversion is greater than zero for some molecular systems as a consequence of the relatively long lifetime of the S_1 state compared to the

5   intersystem crossing rate constant for these systems. Transition from the triplet state 10 back to a singlet state, such as $T_1 \rightarrow S_0$, can occur via the radiative process known as phosphorescent emission of a photon 24. Phosphorescence is generally characterized by a relatively long radiative lifetime compared to fluorescence due to the disallowed nature of the process. An example of single-photon excitation to an allowed energy level 2 is promotion of the dye molecule coumarin

10   from a ground electronic state to an excited electronic state through the absorption of a single photon 4 at 400 nm, followed by internal conversion 16 and subsequent fluorescent emission of a photon 21 at 480 nm. In this example the probability of excitation is linearly related to the power of the incident optical radiation, thus single-photon excitation to an allowed energy level 2 is referred to as a linear excitation process.

15   For the second Jablonski diagram shown in FIGURE 1, single-photon excitation to a virtual energy level 26 occurs upon absorption of a photon 28 having insufficient energy to directly promote the molecule to a higher allowed electronic energy level 8. Instead, the molecule is promoted to a very short lived virtual energy level 30. This virtual energy level 30 will typically have a lifetime on the order of 10^{-15} sec. Virtually instantaneous re-emission 32 of the absorbed

20   photon 28 from this virtual level 30 will typically occur via processes such as elastic scatter. An important example of this process is Rayleigh scatter at 800 nm from coumarin upon excitation

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with light at 800 nm. Another example is Raman scatter, which occurs when the molecule returns to the various vibrational levels associated with the ground state. In these example processes the probability of excitation is also linearly related to the power of the incident optical radiation. thus single-photon excitation to a virtual energy level 26 is also referred to as a linear excitation process.

5   For the final Jablonski diagram shown in FIGURE 1, simultaneous two-photon excitation to an allowed energy level 34 occurs upon simultaneous absorption of a first of two photons 36 and a second of two photons 38. In this case the combined energy of the first of two photons 36 and the second of two photons 38 is sufficient to promote the molecule from a first allowed energy level 6 to a second allowed energy level 8. Typically, the individual energies of neither the first of

10   two photons 36 nor the second of two photons 38 is sufficient to directly promote this or any other allowed electronic transition. Instead, the first of two photons 36 promotes the molecule to a very short lived virtual energy level 30. This is the same virtual energy level as that shown in the second Jablonski diagram. Before re-emission 32 can occur from the virtual energy level 30, the second of two photons 38 immediately promotes the molecule to a second allowed electronic

15   energy level 8. The result is excitation that is equivalent to that achieved using linear single-photon excitation to an allowed energy level 2. Note that the first of two photons 36 and the second of two photons 38 may be of equal or unequal energy. Also, the instantaneous irradiance. or $W m^{-2}$, of the incident excitation light must be relatively high to yield significant efficiency in absorption of the second of two photons 38 before the virtual energy level 30 undergoes relaxation 32 back to the

20   original first allowed electronic energy level 6. In fact, because the lifetime of the virtual energy level 30 is on the order of 10^{-15} sec, pulsed excitation sources having very high peak powers are commonly used to efficiently stimulate these processes; such sources are often preferable since

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they are capable of providing large numbers of photons to the excited molecule during the brief

lifetime of the virtual energy level 30. Once the molecule has been promoted to the second allowed

electronic energy level 8, it can then undergo rapid internal conversion 16, followed by further

relaxation, such as through collisional deactivation 20, fluorescent emission of a photon 21, or

5   intersystem crossing 22 to a triplet state 10. In the last case, transition from the triplet state 10 back

to the singlet ground state 6, can occur via phosphorescent emission of a photon 24. It is notable

that simultaneous two-photon excitation shares features of both single-photon excitation to an

allowed energy level 2 and single-photon excitation to a virtual energy level 26, specifically in that

a virtual energy level 30 plays a key role in the promotion of the molecule from the ground state to

10   the excited state, and that once promoted to an excited energy level the molecule can undergo

photo-chemical and photo-physical processes that are identical to those resulting from single-

photon excitation to an allowed energy level 2. An example of the simultaneous two-photon

excitation process is the promotion of the dye molecule coumarin from a ground electronic state to

an excited electronic state through the simultaneous absorption of two photons at 800 nm, followed

15   by emission of a fluorescent photon at 480 nm. Due to the well known quadratic dependence on

instantaneous photon irradiance, simultaneous two-photon excitation to an allowed energy level 50

is also referred to as a non-linear excitation process. The significant differences between linear

and non-linear excitation processes are identified in the next section.

Note that in addition to the example energy level diagrams shown in FIGURE 1, many

20   other possible transitions and energy level conditions are possible, depending upon numerous

factors, including the characteristics of the molecular system, its environment, and the particular

energies of the absorbed and released forms of energy, along with their temporal and spatial

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correlations. Once a molecule has been promoted to an excited state, a variety of physical or chemical processes may occur, including luminescent emission of a photon. photochemical transformation, such as isomerization or oxidation, or photo-ionization. Importantly, though, it is the fundamental properties of the excited state and its environment that determine the ultimate fate

5   of the molecule. Once excited, the mechanism responsible for promoting the molecule to the excited state has no significant impact on this fate since the excitation process itself does not directly impact the subsequent properties of the excited molecule or its environment. Hence. a molecular diagnostic agent that works well under single-photon excitation conditions may be expected to exhibit similar behavior under two-photon excitation conditions.

10   Comparison of linear and non-linear excitation – power dependence and spatial effects:

When light interacts with a molecular system. it induces a polarization that is proportional to the linear susceptibility multiplied by the magnitude of the applied electric field. When this electric field is very intense, the system cannot be described as easily, and higher order interaction terms must be included in the description of the induced polarization. Simultaneous two-photon

15   excitation is referred to as a non-linear process because it occurs when the electromagnetic fields from two photons combine via these higher order terms, specifically the imaginary portion of the third-order susceptibility, $\chi^{(3)}$", to induce an electronic transition. This is another way of

describing the non-linearity of simultaneous two-photon absorption. That is, the molecular system is reacting non-linearly to the intense electromagnetic field. In contrast, single-photon excitation

20   processes may be described by the linear susceptibility and are linear with excitation power. Note that the cross-section for simultaneous two-photon excitation is typically about one hundred thousand-fold smaller than that for an equivalent single-photon excitation process. This is due to

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the low probability that two photons will simultaneously interact with a molecule during the lifetime of the extremely brief virtual energy level. However, the availability of optical excitation sources capable of providing extremely high peak powers, such as mode-locked lasers, can substantially ameliorate the impact of this low efficiency by increasing instantaneous incident

5   powers and thereby dramatically increasing the efficiency of simultaneous two-photon excitation. For example, when using continuous wave excitation the efficiency of two-photon excitation for a particular molecular system may be 10^5 smaller than that achieved with single-photon excitation. However, if the same average optical power is emitted in the form of a train of very short pulses, the shift in product of the peak and average powers can change this ratio such that it is close to

10   unity.

The non-linear nature of simultaneous two-photon excitation can be exploited to achieve an important difference in the spatial excitation properties of simultaneous two-photon excitation compared to linear excitation. For example, FIGURE 2 shows that the single-photon excitation efficiency profile 40 and the simultaneous two-photon excitation efficiency profile 42 differ

15   dramatically as a function of the beam intensity profile 44 when a laser beam 46 is focused 48 into a material 50. This material 50 might be a laser dye solution held between the walls of a cuvette 52. Another example of this material 50 might be human tissue underneath skin. Focussing 48 of the laser beam 46 with a lens 54 produces a beam intensity profile 44 that varies as a function of distance through the sample 50, reaching a maximum level at the center of the focus 56 as predicted

20   by classical Gaussian optical theory. For a single-photon process, the linear relationship between beam intensity (or incident power) and excitation efficiency results in a single-photon excitation efficiency profile 40 that linearly follows the beam intensity profile 44. In contrast, for the

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simultaneous two-photon process, the non-linear relationship between beam intensity (or incident power) and excitation efficiency results in a simultaneous two-photon excitation efficiency profile 42 that follows the square of the beam intensity profile 44. Hence, focussing 48 the laser beam 46 can be used to substantially limit the extent of excitation to a small focus zone, or confocal region,

5   when simultaneous two-photon excitation is employed. In contrast, when linear excitation is employed, excitation occurs substantially along the entire optical path, making spatial localization of excitation considerably less defined.

Comparison of linear and non-linear excitation – absorption and scattering effects:

While the cross-section for simultaneous two-photon excitation may be considerably lower

10   than that observed with single-photon excitation. use of simultaneous two-photon excitation may be favorable to single-photon excitation under many conditions because of lower matrix absorption and optical scattering of longer wavelength optical radiation. For example, FIGURE 3 shows an absorption spectrum 58 for animal tissue, such as human dermis or liver, covering the ultraviolet (UV) to near infrared (NIR) spectral region. FIGURE 4 shows a scattering spectrum 66 for

15   animal tissue, such as human dermis or liver, under similar conditions. Specifically, FIGURE 3 demonstrates how higher-energy photons 60. such as those used for linear excitation of diagnostic agents, may experience considerably greater tissue absorption than lower-energy photons 62. such as those used for non-linear excitation of diagnostic agents. For instance, human skin strongly absorbs higher-energy photons 60 at 400 nm, but is relatively transparent to lower-energy photons

20   62 at 800 nm. This is a consequence of the relatively high natural absorbance of higher-energy photons 60, having ultraviolet or visible wavelengths, by pigments, proteins, and genetic materials, among other natural components, of skin. Note also the relationship between excitation

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energies and the emission wavelength 64 of the diagnostic agent. Regardless of whether higher-energy photons 60 or lower-energy photons 62 are used to excite the agent, the emission wavelength 64 will occur at an energy that is determined by the agent, not the excitation method applied to the agent. FIGURE 4 further demonstrates how higher-energy photons 68 may

5   experience considerably greater tissue scatter than lower-energy photons 70. Any optically dense medium, such as human skin, will strongly scatter higher-energy photons 68 at visible or ultraviolet wavelengths, for example at 400 nm, but will exhibit much lower scatter for lower-energy photons 70 at NIR or infrared (IR) wavelengths, for example at 800 nm. Note that as shown earlier in FIGURE 3, FIGURE 4 shows that the emission wavelength 72 of the diagnostic

10   agent will typically fall between that of the higher-energy photons 60 and the lower-energy photons 62.

      These differences in optical properties have several important consequences. First, absorption of short-wavelength, higher-energy photons 60 by tissue can result in undesirable tissue damage. In contrast, negligible effects may be experienced under irradiation with lower-energy photons 62, such as NIR light, even when the optical power of the NIR light is many-fold

15   higher than that of the UV or visible radiation. Second, the inherently high absorption and scatter of higher-energy photons 68 by tissue can result in very shallow tissue penetration depths, while lower-energy photons 70 generally have much greater penetration depths. Since scattered higher-energy photons 60 will induce emission from diagnostic agents along their scatter path, higher-

20   energy photons 60 that manage to penetrate tissue will tend to produce a diffuse emission zone that extends perpendicularly to the excitation path; but because of the quadratic dependence on two-photon excitation, irradiation with lower-energy photons 62 will produce a more sharply defined

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excitation pattern that is not significantly blurred by the presence of scattered lower-energy photons 62. Hence, illumination and subsequent detection of subsurface features is difficult or impossible when using higher-energy photons 68, such as those in the UV or visible spectral regions; in contrast, illumination and subsequent detection of subsurface features is much easier when using

5 lower-energy photons 70, such as those in the NIR or IR spectral regions. Note also that the emitted light from the diagnostic agent may be highly absorbed and scattered by the tissue or other optically dense medium under examination. However, for satisfactory detection of the emitted light, it is only necessary that a small fraction of this light make its way to a detector. The large extent to which this emitted light may be scattered implies that sophisticated methods are needed to

10 differentiate emitted light produced by an excited agent from scattered light and other optical or instrumental noise sources. This latter consideration is the topic of a subsequent section.

These important differences in absorption and penetration depth properties for higher-energy and lower-energy light are shown schematically in FIGURE 5. When UV or visible light 74, for example light at 400 nm, impinges on human tissue 76, the majority of the optical energy is

15 immediately absorbed 78 and scattered 80 in the outermost layers 82. such as the epidermis and dermis. Absorption 78 may occur due to excitation of certain molecules in the cells of this tissue 76, such as those composing the genetic material in the cellular nucleus, and can initiate a variety of collateral photochemical changes in these cells at the site of this absorption 78. These collateral photochemical changes can include irreversible genetic damage and induction of cancer. Hence,

20 optical penetration depth is low and potential for induction of collateral damage is high for excitation with UV or visible light 74, such as that conventionally used for linear excitation of diagnostic agents. In contrast, NIR or IR light 84, for example at 800 nm, will experience much

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lower absorption and scatter 80 by tissue 76. The overall depth of penetration will be much greater and the extent of collateral damage to cells will be substantially lower. Hence, if long-wavelength excitation light is used in a two-photon excitation process to replace higher-energy, single-photon excitation, it becomes possible to photo-activate specific diagnostic agents present in deep tissues

5   using relatively non-damaging wavelengths that have high penetration depths.

Furthermore, the salient properties of non-linear excitation shown in FIGURE 2 have additional implications when coupled with the inherent non-damaging nature and high penetration depths possible with the use of NIR light. For example, FIGURE 6 compares the penetration depth and spatial localization characteristics expected for single-photon excitation 86 and

10   simultaneous two-photon NIR excitation 88 of imaging agents present in a subcutaneous tumor 90. Single-photon excitation 86 produces an excitation zone 92 that extends substantially along the entire optical path and has no significant specificity. Note that the efficiency of single-photon excitation 86 will vary along the optical path due to absorption and scatter, being highest 94 near the point of introduction of optical radiation and dropping off rapidly 96 along the optical path.

15   Note also that the potential for induction of collateral photodamage will follow this same trend. Hence, single-photon excitation produces an extended excitation zone 92 that cannot be effectively limited to a finite volume, especially in deep tissues. Also, significant collateral damage can occur throughout surrounding tissues 98, and especially in surface tissues 100. If the single-photon excitation 86 is focussed, the excitation zone 92 will be slightly enhanced at the focus 102. Note,

20   however, that this excitation zone 92 might not even extend all the way into the tumor 90 if the UV or visible light used for single-photon excitation 86 is significantly absorbed or scattered prior to reaching the tumor 90. In contrast, use of NIR simultaneous two-photon excitation 88 produces a

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sharply defined remote excitation zone 104 that is substantially localized to the focus 106 as a consequence of the intrinsic non-linear properties of this excitation method. Furthermore, because of the reduced absorption of NIR light, collateral damage to the surrounding tissues 98 and especially to surface tissues 100 is minimized. And as a consequence of the combined low

5   absorption and scatter of NIR light, it is possible to effectively probe far deeper locations than those feasible using UV or visible wavelengths.

Examples of linear and non-linear excitation of typical diagnostic imaging agents:

Linear excitation of a diagnostic agent in solution is shown in FIGURE 7. In this example, laser radiation at 442 nm was used to excite a dilute solution of the dye molecule FITC in

10   methanol. The laser beam emitted from a continuous wave helium-cadmium laser was focused through a 20x microscope objective into a cuvette containing the dye solution, and stimulates a diffuse, elongated emission pattern in the dye. This example clearly shows that emission occurs along the entire optical path, and that a diffuse halo attributable to stimulation of the dye by scattered laser light surrounds the primary excitation path. In contrast, FIGURE 8 demonstrates

15   highly localized, remote photo-activation of a diagnostic agent using simultaneous two-photon excitation. In this example. laser radiation at 730 nm was used to excite a dilute solution of the dye molecule coumarin 480 in methanol. Specifically, the NIR output of a mode-locked titanium:sapphire laser, which emitted a continuous train of 730 nm wavelength, <200 fs pulses of light at a 78 MHz pulse repetition frequency in a beam approximately 1 mm in diameter, was

20   focused through the same 20x microscope objective into a cuvette containing the dye solution. FIGURE 8 clearly shows that fluorescence response from the dye molecule is limited to the focus of the NIR beam. Because of the quadratic relationship between two-photon excitation and

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instantaneous laser power, stimulation at positions along the excitation path prior to and following the focus is negligible. Also, no halo is observed. although a minor artifact attributable to overexposure of the photographic film is seen in this photograph around the emission zone.

Highly localized remote photo-activation of a diagnostic agent present throughout an optically dense medium is demonstrated in FIGURE 9. This shows a photograph of two-photon excited fluorescence of the dye molecule coumarin 480 distributed evenly throughout a tissue phantom consisting of a block of agarose gelatin. NIR output of the mode-locked titanium:sapphire laser, which emitted a continuous train of 730 nm wavelength, <200 fs pulses of light at a 78 MHz pulse repetition frequency in a beam approximately 1 mm in diameter, was expanded to produce a collimated beam approximately 50 mm in diameter using a beam expanding telescope. This expanded beam was then focused into the gelatin block using a 100 mm focal length, 50 mm diameter biconvex singlet glass lens. The gelatin block was then positioned such that the focus of this 100-mm f.l. lens fell at a position 40 mm into the block. FIGURE 9 clearly shows that fluorescence response from the coumarin 480 is only stimulated at the focus of the NIR beam. Because of the quadratic relationship between two-photon excitation and instantaneous laser power, stimulation at positions along the excitation path prior to and following the focus is negligible. Hence, little or no excitation or collateral photo-activation of damage can occur outside the focus region. Also, because the NIR excitation light is only weakly scattered by the gelatin. sharp focus is maintained at deep penetration depths into the block. Note that the sharpness of the focus is determined by Gaussian optical properties; hence, the length of the confocal region is easily adjusted by changing the optical parameters used for beam expansion and subsequent re-focusing.

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Similar results are obtained if an equivalent excitation process is applied to a labeled tumor specimen, as shown in FIGURE 10. This shows a photograph of two-photon excited fluorescence of the dye molecule coumarin 480 distributed evenly throughout a block of mouse carcinoma tissue. As in FIGURE 9, a tightly localized site of activation is demonstrated, even for

5   this sample having an extremely high optical density.

Excitation sources for two-photon excitation of diagnostic imaging agents:

The relatively low cross-section for simultaneous two-photon excitation, which is typically about one hundred thousand-fold smaller than that for an equivalent single-photon excitation process, means that special optical excitation sources must typically be used to efficiently excite

10   diagnostic agents. Optical sources that provide high peak powers can be used to substantially ameliorate the impact of this low efficiency by increasing instantaneous incident powers while maintaining modest average power levels. In fact, quasi-continuous wave mode-locked lasers, such as the mode-locked titanium:sapphire laser, are ideal for exciting molecular diagnostic agents in optically dense specimens, such as biological tissues. Specifically, such lasers are capable of

15   delivering NIR peak powers in excess of 10 kW, but in the form of very high repetition rate (> 25 MHz pulse repetition rate), ultra-short (~ 200 fs pulse duration), low energy (~ 1 nJ per pulse) pulses; partitioning of average laser power (on the order of 10 mW to 2 W) into a high frequency train of ultra-short pulses yields an excitation beam that is extremely efficient for stimulating two-photon excited fluorescence but is essentially harmless to biological materials. The quasi-

20   continuous output of mode-locked or other high-repetition rate lasers is also highly compatible with various modulation methods, especially when the modulation is performed at frequencies considerably below the pulse repetition frequency of the laser, since the pulsed nature of the source

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can be ignored in the subsequent demodulation process.

The specific example of the mode-locked titanium:sapphire laser is continuously tunable over a wavelength band extending from approximately 690 nm to 1080 nm, which corresponds well to a region of minimal scatter and absorption for biological specimens. Two-photon

5　　absorption in this band also corresponds to an important single-photon absorption region, from 345 nm to 540 nm, for many possible diagnostic imaging agents; while two-photon selection rules are sometimes quite different from corresponding single-photon selection rules, strong absorption for the single-photon process can be indicative of significant two-photon absorption at wavelengths approximately twice that of the single-photon wavelength.

10　　It will be clear that, in addition to the mode-locked titanium:sapphire laser, various other optical sources are applicable for excitation of diagnostic imaging agents. Especially important are diode lasers, Nd:YAG and Nd:YLF lasers, and optical parametric oscillators, amplifiers and generators. Pulsed diode lasers offer attractive performance as a result of their extremely high operational efficiencies, and are available at a variety of wavelengths in the NIR. Mode-locked

15　　Nd:YAG and Nd:YLF lasers provide an efficient, reliable means for generating NIR excitation light at 1064 nm and at 1047 or 1053 nm, respectively. Mode-locked optical parametric oscillators, amplifiers and generators are capable of producing optical radiation covering a band from approximately 500 nm to greater than 3000 nm; availability of wavelengths from 1000 nm to 1800 nm affords a practical means for exciting diagnostic agents using light in a band of

20　　exceptionally low tissue scatter and absorption, and may be especially useful for activation of NIR diagnostic agents (ie, those that have single-photon absorption bands at wavelengths in excess of 500 nm). Also, various other pulsed or mode-locked lasers have applicability, including: argon

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ion lasers; krypton ion lasers; helium-neon lasers; helium-cadmium lasers; ruby lasers; Nd:YAP,

Nd:YVO4, Nd:Glass, and Nd:CrGsGG lasers; regeneratively amplified lasers; Cr:LiSF lasers;

Er:YAG lasers; F-center lasers; Ho:YAF and Ho:YLF lasers; and copper vapor lasers. Various

continuous wave lasers may also be used, but with considerably lower efficiency than that

5   achieved using pulsed lasers.

Detection of two-photon excited emission from diagnostic imaging agents:

Spatial information concerning the origin of the emitted light from a two-photon excited

diagnostic imaging agent is encoded by and may be correlated to the excitation focus. This is in

stark contrast with single-photon excited imaging methods, including those based on photon

10   migration, where the diagnostic imaging signal must be carefully deconvolved from emission light

generated along the entire excitation path and from emission produced by scattered excitation light.

Hence, it is not necessary for the light emitted from the two-photon excited diagnostic agent to be

detected or imaged directly without scatter. In fact, it is only necessary that a fraction of this

emitted light be collected and detected in such a way that the collection and detection process does

15   not distort the correlation between detected signal and emission point of origin.

To understand the significance of the relationship between signal detection and two-photon

excited emission point of origin, it is useful to consider what happens to the emitted light

immediately following the instant of emission. When imaging in an optically dense specimen,

such as biological tissue, light from the two-photon excited diagnostic imaging agent will be

20   emitted in an essentially isotropic manner. Some fraction of this emitted light will travel directly to

a detector apparatus mounted remotely from the point of emission, while some other fraction will

travel a circuitous route to the detector apparatus as a consequence of one or more scattering events

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occurring between emission and detection. If an attempt is made to image at a depth of 10 cm in a

biological specimen, the transit time for an unscattered, or ballistic, emitted photon (that is, the total

transit time from instant of emission to exit from a surface of the specimen) will be approximately

0.3 ns: for a highly scattered emitted photon, this transit time could be as high as 3-10 ns. Thus,

5   for maximum efficiency in this example, it would be desirable to integrate all of the emitted light

for a period of time sufficient to capture most or all of the ballistic and highly scattered photons.

This implies that for imaging at depths of 10 cm or less, an integration period of approximately 10

ns would be appropriate.

If an image is to be generated by moving or scanning the location of the excitation focus

10   relative to the specimen, the foregoing analysis implies that the excitation point should not be

moved more frequently than once every 10 ns. In fact, practical limitations on scanning processes

and mechanisms, combined with signal-to-noise arguments concerning minimum dwell times and

the additional possible use of modulation methods, mandate that scanning be performed using

dwell times in excess of 1 μs. Thus, for intensity based imaging with dwell times in excess of 1

15   μs and possible modulation frequencies of 1 MHz or less, it makes little difference where the

detector is located as long as it is situated such that it can collect a significant portion of the ballistic

and scattered emitted light (the choice of location of detector relative to the emission point of origin,

and hence the length of time introduced due to optical delay, has little or no effect on the ability to

correlate the detected signal with its origin because of the short transit time relative to other

20   measurement parameters). Accordingly, it will be clear that the detector may be located in such a

way that it comprises an epi-illumination configuration with the excitation beam, or that it may be

located externally to the excitation beam. It is notable that the epi-illumination configuration (or

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other possible co-linear excitation and detection configurations) minimizes potential parallax losses for detection of surface or near surface objects, but that such configurations are more susceptible to interference from elastically scattered or reflected excitation light. Parallax losses may be minimized for external detection configurations by actively orienting the detection system such that

5   it maintains consistent registry with the point of excitation, by using multiple detection assemblies that are individually optimized for collection of emitted light from different zones within the specimen, or by locating the detection system sufficiently far from the specimen such that parallax losses are minimal.

The discussion on detection of emitted light from two-photon excited diagnostic imaging

10   agents has focused to this point on intensity based methods, wherein an image may be constructed by correlating detected intensity of emission with location of excitation for multiple excitation points throughout a specimen. However, intensity based methods are not always optimal, since they are susceptible to a number of complicating factors, including:

- Variations in scatter and absorption of excitation light due to heterogeneities in the specimen

15   – heterogeneities, such as areas of abnormal optical density, that are located between the excitation source and the intended point of excitation can translate into unanticipated differences in effective excitation level at the intended point of excitation. Artifacts caused by this phenomenon can be ameliorated by acquiring data along several excitation paths that are affected to different extents by this heterogeneity, followed by subsequent

20   deconvolution of the resultant multiple data sets, but this may be difficult or impossible for some specimens.

- Variations in scatter and absorption of emitted light due to heterogeneities in the specimen –

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heterogeneities, such as areas of abnormal optical density, that are located between the point of emission and the detection system can translate into unanticipated differences in collection efficiency for light emitted from the point of excitation. Artifacts caused by this phenomenon can be ameliorated by acquiring data along several collection paths that are

5   affected to different extents by this heterogeneity, followed by subsequent deconvolution of the resultant multiple data sets, but this may be difficult or impossible for some specimens.

- Variations in concentration or local environment of diagnostic imaging agents that are not directly correlated with form or function – it is assumed in intensity based imaging that changes in emission level throughout a specimen can be correlated with structural or

10   physiological organization of the specimen. However, if the imaging agent is not appropriately distributed throughout the specimen, or if other factors, such as heterogeneity in the local environment within the specimen, affect the emission of the imaging agent in ways that cannot be correlated with form or function, then it becomes harder to obtain meaningful data from the specimen. Artifacts caused by this phenomenon can be

15   ameliorated by using or by designing imaging agents that are not susceptible to such factors, but this may be difficult or impossible for some specimens.

A detection approach that is less susceptible to optical heterogeneity of the specimen could be based on measurement of change in excited state lifetime rather than on intensity of emission. Excited state lifetimes are an intrinsic property of the excited state of a molecular agent and its

20   immediate environment, and fortuitously the accurate measurement of lifetimes are immune to all but the grossest variations in excitation level and collection efficiency. A convenient means for measuring excited state lifetimes uses phase photometric methods to correlate phase shift between a

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modulated excitation source and the resultant emission signal to lifetime. Specifically, the

preceding discussion on photon transit times implies that phase photometric methods are applicable

for imaging in optically dense media, especially for agents with lifetimes in excess of 1-10 ns.

Hence, if diagnostic imaging agents are used that have emission lifetimes that correlate with form

5   or function within the specimen, such as quenching of fluorescence of an imaging agent in the

presence of oxygen or concentration of an imaging agent within a structure, then imaging based on

change in lifetime rather than on emission intensity becomes practical. Such lifetime based

methods would have equal applicability to laser scanning microscopy and to remote imaging of

extended objects, such as a tumor in a human subject.

10   Appropriate collection devices for transduction of intensity or phase based emission data

include, but are not limited to, photomultiplier tubes, microchannel plate devices, photodiodes,

avalanche photodiodes, charge coupled devices and charge coupled device arrays, charge injection

devices and charge injection device arrays, and photographic film.

<u>Noise reduction methods for recovery of two-photon excited emission from diagnostic imaging</u>

15   <u>agents – modulation and second harmonic detection</u>:

The inherently low efficiency of the two-photon excitation process can translate into a very

high ratio of scattered, unabsorbed excitation light to two-photon excited fluorescence emission.

Furthermore, the importance of other possible linear interferences attributable to this very high

excitation level, including single-photon excited fluorescence of the agent or other species present

20   in the specimen under examination, Raman scatter, and other phenomena, along with the need to

eliminate interferences from ambient light and other optical or electronic noise sources, all indicate

that a modulated excitation method coupled with appropriate demodulation of the detector signal

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should provide optimal discrimination against interferences and enhanced recovery of the analytical signal. In fact, interferences from background reported by Denk et al. (U.S. Patent No. 5,034,613) could be largely circumvented if suitable modulation and demodulation methods were used, including demodulation at the pulse repetition frequency of the laser; use of such methods

5   would dramatically improve signal-to-noise (SNR) performance of their microscope. In general, modulation can improve detection performance for virtually any measurement in one or more ways:

(1) Rejection of continuous background or noise sources – in the example of Denk's two-photon laser scanning microscope, modulation of the excitation source with subsequent demodulation of the detector signal, using a device such as a lock-in amplifier (LIA) or a

10   heterodyne demodulator, would limit detection system response to a band of frequencies closely related to the modulation frequency. By controlling the phase sensitivity of this demodulation, additional discrimination would be achieved against signals that are not linked to or closely matched with the modulation pattern. Hence, by suitable selection of modulation frequency and demodulation phase, interferences from noise sources such as room light or electronic noise at

15   specific frequencies, for example from a nearby electric motor, can be strongly rejected. This approach is equally valid for remote imaging of extended objects, such as a tumor in a human subject.

(2) Rejection of broadband or "pink noise" sources – the measurement environment, along with the electronics and other devices used for any measurement, contribute broadband noise,

20   sometimes called pink noise, into any measurement. The impact of this intrinsic noise can be greatly reduced through the use of bandwidth-limited detection methods. Specifically, for a given optical measurement, the observed signal voltage, V_{SIGNAL}, is related to a detector input current,

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i_{INPUT}, produced by photons interacting with a detector, multiplied by the input impedance, Z_{INPUT}, and the gain of the detection system, G, according to the following:

$$V_{SIGNAL} = i_{INPUT} \cdot Z_{INPUT} \cdot G, \tag{1}$$

5

while the observed noise voltage, V_{NOISE}, may be approximated by the product of the noise current, i_{NOISE}, the input impedance, the square root of the electronic or optical bandwidth, B, of the detection system, and the gain, according to following:

$$V_{NOISE} = i_{NOISE} \cdot Z_{INPUT} \cdot B^{1/2} \cdot G. \tag{2}$$

Hence SNR may be estimated from the ratio of these two voltages, (V_{SIGNAL} / V_{NOISE}). When a typical optical detector, such as a photomultiplier tube (PMT), is used to detect an unmodulated fluorescence signal, this detector will produce a certain signal level along with a noise current. For an example PMT, such as the Hamamatsu R928 (7.4×10^5 A/W radiant anode sensitivity), an optical input at a level of 10 pW produces 7.4 μA i_{SIGNAL}. If this signal current is converted to voltage in a low noise amplifier having a gain of 100, an input impedance of 50 Ω. an input noise level of 5 nV/$\sqrt{Hz}$, and a bandwidth of 1 MHz, the following signals are produced:

20 $\qquad V_{SIGNAL} = 7.4 \, \mu A \cdot 50 \, \Omega \cdot 100 = 37 \, mV;$

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$$V_{\text{NOISE}} = 5 \text{ nV/}\sqrt{\text{Hz}} \cdot (10^7 \text{ Hz})^{1/2} \cdot 100 = 1.6 \text{ mV}.$$

Note that Ohm's Law, or $V = i \cdot R$, has been substituted for noise current and impedance shown

in Eq. 2. Thus, for this broadband example, $SNR = 23$. If this excitation energy is modulated,

5   for example sinusoidally at 1 MHz with a 100% depth of modulation, the value of V_{SIGNAL} will

decrease to approximately 18.5 mV (assuming that this modulation is introduced by cyclic

attenuation or other loss-based modulation method that results in an overall loss of 50% of average

power without changing peak excitation power). But if the detection system uses bandwidth

limited demodulation at 1 MHz having a bandwidth of 1 kHz, the pink noise decreases far faster

10   than the signal:

$$V_{\text{NOISE}} = 5 \text{ nV/}\sqrt{\text{Hz}} \cdot (10^3 \text{ Hz})^{1/2} \cdot 100 = 16 \, \mu\text{V},$$

and the overall SNR increases to approximately 1200. Thus, although some signal strength is lost

15   when using many forms of modulation, the overall increase in SNR more than compensates for

this loss. Further, if there is any linear interference in the detector response, for example from

ambient light leakage into the detector, the broadband detection scheme will detect this as an

additional noise source, while the modulated, bandwidth limited scheme will reject this

interference. Assume that ambient leakage produces a background signal of 1 μA on the PMT,

20   which translates to 5 mV of background signal. For the unmodulated case, optical shot noise from

this background, B, is equal to the square root of the total photons detected, and $SNR \approx S/(S +$

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$B)^{1/2}$; this yields an estimated SNR of approximately 5.7. Notably, the SNR for the modulated

case is essentially unchanged. This analysis is equally applicable to laser scanning microscopy and

to remote imaging of extended objects, such as a tumor in a human subject.

(3) Rejection of linear interferences at the modulation frequency – as a consequence of the

5 inherently low efficiency of the two-photon excitation, the ratio of scattered, unabsorbed excitation

light to two-photon excited fluorescence emission is generally quite high. This includes linear

interferences at the modulation frequency that arise from elastic and inelastic scatter as well as from

single-photon excited fluorescence. Optical filtering is frequently used in an effort to spectrally

distinguish two-photon emission from these optical background phenomena. Unfortunately, these

10 interferences can be exceedingly difficult or impossible to eliminate using spectral means alone. As

an alternative to ignoring these residual interference sources, one common approach for recovery

of pure two-photon signal utilizes regression of the detected signal at several excitation power

levels against excitation power level, so that the quadratic two-photon excited fluorescence

component can be extracted mathematically from linear interferences; this makes use of a model of

15 total fluorescence response, I_f, given by:

$$I_f = \alpha\, I_L + \beta\, I^2_L \tag{3}$$

where I_L is the instantaneous excitation intensity, α is a proportionality constant for various linear

20 effects, and β is a proportionality constant for two-photon excited fluorescence. While this

regression-based method is appropriate for laboratory use where the necessary number of

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measurements per unit of time is small, it is too time consuming, complicated, and impractical whenever total data acquisition time must be minimized, such as in the case of multiple point scanned optical imaging. Far faster results can be obtained through the use of temporal rejection methods, such as second harmonic detection, which eliminates the need for performing multiple

5 measurements at several power levels. Freeman et al. (R.G. Freeman, D.L. Gilliland and F.E. Lytle, "Second Harmonic Detection of Sinusoidally Modulated Two-Photon Excited Fluorescence," Analytical Chemistry, 62 (1990) 2216-2219) teach of second harmonic detection methods useful for the analysis of chemical samples, wherein sinusoidal modulation of the excitation source is used to generate a signal at twice the modulation frequency that is related only

10 to two-photon excited fluorescence. A lock-in amplifier referenced to the modulation frequency is used to recover the pure two-photon signal at the second harmonic of the modulation frequency. While the second harmonic fluorescence signal is only approximately 12% of the total two-photon fluorescence produced, the improved rejection of linear interferences more than compensates for the loss in absolute signal level, resulting in an increase in the overall SNR. Hence, the second

15 harmonic detection method is ideally applicable to laser scanning microscopy and to remote imaging of extended objects, such as a tumor in a human subject, as a consequence of its intrinsic efficiency in rejection of scatter and its high data bandwidth potential. These advantages mean that an imaging system using second harmonic detection can reliably obtain pure two-photon excited emission signals with minimal dwell times at each point, and with use of maximum excitation

20 power for each measurement at each point.

The preceding enumerated advantages for the use of modulation methods in two-photon excited diagnostic imaging apply equally well whether data is acquired based on measurement of

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emission intensity or excited state lifetime. In fact, lifetime measurements are most readily and sensitively measured using phase photometric methods that are based on determination of phase shifts between a modulation waveform and the detected signal. Hence, it is clear that modulation methods, including those based on second-harmonic detection, have important utility in the

5   efficient detection of two-photon excited fluorescence, where they serve to eliminate interferences from ambient and instrumental noise sources as well as from scattering and other phenomena occurring within the specimen undergoing examination. For optically dense media, such as human tissue, the extremely high ratio of scattered, unabsorbed excitation light to two-photon excited fluorescence emission makes use of such methods vital. Hence, for clinical imaging applications

10   or for two-photon laser scanning microscopy, employment of modulation methods as described here will always be advantageous.

Contrast agents in two-photon excited imaging – endogenous and exogenous agents:

The foregoing discussion has shown that non-linear two-photon excitation can be used to effect important improvements in the specificity and depth of penetration for optically excitable

15   molecular agents present in optically dense media, and that detection performance can be improved by use of encoding and decoding methods on the respective excitation and detection processes. The exceptional spatial localization of excitation possible when using two-photon methods can be harnessed to significantly improve contrast in the point of excitation. Once this localized excitation is effected, the analytic light thereby emitted may be detected using a variety of detection means. If

20   this excitation point is caused to move relative to the specimen under examination, for example by scanning the position of the focus relative to the specimen or by scanning the position of the specimen relative to the focus, then a two- or three-dimensional image of the specimen can be

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generated by making a correlation between the location of the excitation point and the emitted light thereby produced. Useful contrast in this image, however, also depends on the existence of differences in the concentration or local environment of the molecular agent or agents responsible for emission. These agents may be endogenous or exogenous to the specimen, and imaging is

5   ultimately based on contrasts in their localized emission properties that can be correlated to heterogeneity in structure or function within the specimen. Hence, it is important to also carefully consider the role of these contrast agents in non-linear diagnostic imaging.

Various endogenous chromophoric agents may be useful for diagnostic imaging, particularly of diseased tissue. Because of structural or physiological differences between diseased

10   and non-diseased tissues, between various internal substructures and organs in higher animals, or between different ranges of healthy or sub-healthy tissues, the concentration or local environment of natural chromophoric agents, such as aromatic amino acids, proteins, nucleic acids, cellular energy exchange stores (such as adenosine triphosphate), enzymes, hormones, or other agents, can vary in ways that are useful for probing structural or functional heterogeneity. Thus, these

15   endogenous indicators of heterogeneity can be probed non-invasively using two-photon excitation.

Unfortunately, in many cases the specificity possible with such agents is inadequate to achieve meaningful diagnostic imaging, and so exogenous agents must be added to the specimen. Traditional exogenous agents semi-selectively partition into specific tissues. organs, or other structural units of a specimen following administration. The route for administration of these

20   agents is typically topical application or via systemic administration. Under ideal conditions, these agents will partition into or otherwise become concentrated on or in the structures of interest, or may be excluded preferentially from these structures. This concentration may be a consequence of

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isolated topical application directly onto a superficial structure, or through intrinsic differences in the physical or chemical properties of the structure which lead to partitioning of the agent into the structure. Contrast between areas of high concentration and low concentration can thereby be used as a basis for probing structural or physiological heterogeneity. Alternatively, exogenous agents

5 may permeate throughout a specimen; if their emission properties, such as chromatic shift, quenching, or lifetime, are sensitive to physiological heterogeneity, then these parameters of the contrast agent can be used as the basis for contrast in imaging.

Because the emission properties of a molecular agent are determined by the fundamental properties of the excited state and its environment, the mechanism responsible for promoting the

10 agent to the excited state has no significant impact on the emission properties of the excited state. Hence, a molecular diagnostic or contrast agent that works well under single-photon excitation conditions may be expected to exhibit similar behavior under two-photon excitation conditions. In general, any contrast agent that is useful for single-photon excitation can be used with two-photon excitation, where the enhanced control over site of excitation will serve to improve resolution of the

15 image. Appropriate contrast agents include many molecular agents used as biological dyes or stains, as well as those used for photodynamic therapy (PDT). Standard PDT agents have tissue specificities that in general are based on the combined chemical and physical properties of the agent and the tissue, such as a cancerous lesion. These agents are efficient absorbers of optical energy, and in many cases are luminescent. For example,

20 • psoralen and its derivatives (including 5-methoxypsoralen [or 5-MOP]; 8-methoxypsoralen [8-MOP]; 4,5',8-trimethylpsoralen [TMP]; 4'-aminomethyl-4,5',8-trimethylpsoralen [AMT]; 4'-hydroxymethyl-4,5',8-trimethylpsoralen [HMT]; 5-chloromethyl-8-methoxy-

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psoralen, Angelicin [isopsoralen]; 5-methlyangelicin [5-MIP]; and 3-carbethoxypsoralen);

- various porphyrin and hematoporphyrin derivatives (including haematoporphyrin derivative [HPD]; Photofrin II; benzoporphyrin derivative [BPD]; protoporphyrin IX [Pp IX]; dye hematoporphyrin ether [DHE]; polyhematoporphyrin esters [PHE]; 13,17-N,N,N-dimethylethylethanolamine ester of protoporphyrin [PH1008]; tetra(3-hydroxyphenyl)-porphyrin [3-THPP]; tetraphenylporphyrin monosulfonate [TPPS1]; tetraphenylporphyrin disulfonate [TPPS2a]; dihematoporphyrin ether; meso-tetraphenyl-porphyrin; and mesotetra(4N-methylpyridyl)porphyrin [T4MPyP]) along with various tetraazaporphyrins (including octa-(4-*tert*-butylphenyl)-tetrapyrazinoporphyrazine [OPTP]; tetra-(4-*tert*-butyl)phthalocyanine [t₄-PcH₂]; and tetra-(4-*tert*-butyl)phthalocyanato-magnesium [t₄-PcMg]);

- various phthalocyanine derivatives (including chloroaluminum-sulfonated phthalocyanine [CASPc]; chloroaluminum phthalocyanine tetrasulfate [AlPcTS]; mono-, di-, tri- and tetra-sulphonated aluminum phthalocyanines [including AlSPc, AlS2Pc, AlS3Pc and AlS4Pc]; silicon phthalocyanine [SiPc IV]; zinc(II) phthalocyanine [ZnPc]; bis(di-isobutyl octadecylsiloxy)silicon 2,3-naphthalocyanine [isoBOSINC]); and Ge(IV)-octabutoxy-phthalocyanine;

- various rhodamine derivatives (including rhodamine-101 [Rh-101]; rhodamine-110 [Rh-110]; rhodamine-123 [Rh-123]; rhodamine-19 [Rh-19]; rhodamine-560 [Rh-560]; rhodamine-575 [Rh-575]; rhodamine-590 [Rh-590]; rhodamine-610 [Rh-610]; rhodamine-640 [Rh-640]; rhodamine-6G [Rh-6G]; rhodamine-700 [Rh-700]; rhodamine-800 [Rh-800]; rhodamine-B [Rh-B]; sulforhodamine 640 or 101; and sulforhodamine B);

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- various coumarin derivatives (including coumarin 1, 2, 4, 6, 6H, 7, 30, 47, 102. 106. 120, 151, 152, 152A, 153, 311, 307, 314, 334, 337, 343, 440, 450, 456, 460. 461, 466, 478, 480, 481, 485, 490, 500, 503, 504, 510, 515, 519, 521, 522, 523, 535, 540, 540A, 548);

- various benzophenoxazine derivatives (including 5-ethylamino-9-diethylaminobenzo[a]-phenoxazinium [EtNBA]; 5-ethylamino-9-diethylaminobenzo[a]phenothiazinium [EtNBS]; and 5-ethylamino-9-diethylaminobenzo[a]phenoselenazinium [EtNBSe]);

- chlorpromazine and its derivatives;

- various chlorophyll and bacteriochlorophyll derivatives (including bacteriochlorin a [BCA]);

- various metal-ligand complexes, such as tris(2,2'-bipyridine)ruthenium (II) dichloride (RuBPY);

- pheophorbide a [Pheo a]; merocyanine 540 [MC 540]; Vitamin D; 5-amino-laevulinic acid [ALA]; photosan; chlorin e6, chlorin e6 ethylenediamide, and mono-L-aspartyl chlorin e6; pheophorbide-a [Ph-a]; phenoxazine Nile blue derivatives (including various phenoxazine dyes);

- various charge transfer and rediative transfer agents, such as stilbene, stilbene derivatives and 4-(N-(2-hydroxyethyl)-N-methyl)-aminophenyl)-4'-(6-hydroxyhexylsulfonyl)stilbene (APSS); and

- numerous other photo-active agents,

will in general become accumulated either at or near a point of application or semi-selectively within a specific tissue due to differences in the physical or chemical properties of the tissue which

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lead to partitioning of the PDT agent into the tissue; once accumulated, such agents will be susceptible to two-photon excitation, and their luminescent or other emission properties can used for acquisition of imagery data. Other photoactive agents that absorb light and are capable of subsequent energy transfer to one or more other agents may also be used, either alone or in

5   conjunction with one or more responsive agents that are capable of accepting this transferred energy and transforming it into a radiative emission.

Biogenic contrast agents in two-photon excited imaging:

Under ideal conditions, standard contrast agents derive target specificity based on chemical or physical affinity for specific tissues. In this way, contrast agents partition into or otherwise

10   become concentrated on or in tissues of interest. Unfortunately, this target specificity is usually not perfect. In fact, it is desirable to have an improved method for increasing specificity in the targeting of agent destination. A means for achieving such improvement in specificity is based on utilization of specific biological signatures of structure, function, or disease. For example, by coupling anti-sense oligonucleotide agents to one or more photo-active moieties. such as FITC.

15   new biogenic contrast agents are created that are capable of selectively tagging only specific cells, such as cancerous cells, that contain complementary genetic encoding. Moreover, the basic approach is easily extended to numerous genetic-based diseases or other disorders by changing the oligomeric code used for the biogenic probe. Employment of two-photon activation enables this powerful approach to be applied using the combined bio-specificity of the biogenic probe and the

20   high spatial localization inherent to the simultaneous two-photon photo-activation process. Thus, very high contrast, very high resolution imaging becomes possible at the genetic level using agents that are specifically targeted for a particular organ, tissue, or lesion.

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An optimal design for biogenic probes utilizes one or more photo-active moieties that have emission properties that change upon complexation between the biogenic agent and the target site. Specifically, changes in emission wavelength or lifetime upon complexation can be used to increase sensitivity of the general method, since such changes will help to increase contrast

5 between areas containing complexed agent and those containing uncomplexed agent. An example is a biogenic agent based on a photo-active moiety that is quenched until complexation occurs, upon which occurrence emission becomes unquenched. Another example is an agent based on an intercalating photo-active moiety, such as psoralen, that is tethered to an anti-sense genetic sequence; upon complexation between the anti-sense sequence and its target sequence,

10 intercalation of the photo-active moiety is enabled that leads to a chromatic shift in emission properties of the photo-active moiety.

It will be clear from the foregoing discussion that targeting methods based on other bio-specific means, such as immunological means, rather than solely on genetic means, are also covered within the scope of the invention. Specifically, agent specificity based on antigen-

15 antibody methods, where an antibody probe is coupled to a photo-active group, provides a powerful new means for diagnosis of disease and infection. Additional means for achieving biospecificity in agent targeting include, but are not limited to, use of ligands, haptens, carbohydrate, lipid, or protein receptors or complexing agents, chelators, and encapsulating vehicles, such as liposomes, fullerenes, crown ethers, and cyclodextrins.

20 FIRST EXEMPLARY EMBODIMENT OF THE INVENTION:

Hence, it is a specific preferred embodiment of the subject invention to employ the output of a NIR source to induce simultaneous two-photon photo-activation of endogenous or exogenous

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diagnostic imaging agents present in a specimen using light at a wavelength approximately twice that necessary for conventional single-photon photo-activation. This preferred embodiment is shown in FIGURE 11. The NIR Source 108 produces a beam of NIR radiation 110 consisting of a rapid series of high peak power pulses of NIR radiation. For example, standard commercially

5   available mode-locked titanium:sapphire lasers are capable of outputting mode-locked pulses with durations <200 fs and pulse energies of about 20 nJ at pulse repetition frequencies in excess of 75 MHz; this source produces a quasi-continuous beam of light having a relatively low average power (up to several Watts) but high peak power (on the order of 100 kW) that is continuously tunable over a NIR wavelength band from approximately 690-1080 nm. The pulse train emitted by

10   the NIR source 108 constitutes a beam of NIR radiation 110 that is easily focussed using standard optical means, such as reflective or refractive optics 112. The focused NIR beam 114 can then be directed onto a specimen 116 to be imaged. Simultaneous two-photon photo-activation of the diagnostic imaging agent will be substantially limited to the confocal region 118 of the focused beam 114 due to the high instantaneous irradiance level that is only present at the focus. Excitation

15   light that is scattered 120 by the specimen 116 will not have a sufficient instantaneous irradiance level for significant excitation of any diagnostic imaging agent that may be present in areas outside of the confocal region 118. Light emitted 122 by diagnostic imaging agent molecules present in the confocal region 118 will exit the confocal region 118 in a substantially isotropic manner. A portion of the emitted light 124 is captured by a detection means 126, such as a photomultiplier

20   tube, that is mounted at a position inside or outside of the specimen 116. This detection means 126 is fitted with a wavelength selection means 128, such as an optical bandpass filter, that serves to pre-process the captured portion of the emitted light 124 in such a way that the selection means 128

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rejects a major fraction of the elastically scattered light while passing a major fraction of light at the

wavelength or wavelengths corresponding to that which is principally characteristic of emission

from the diagnostic agent. The signal thus issued 130 from the detection means 126 is captured by

a processor means 132, the primary purpose of which is to record emission response from

5   diagnostic imaging agent as a function of location of the confocal region 118. By causing the

location of the confocal region 118 to be scanned throughout the volume of the specimen 116, a

complete image of the specimen 116 may be obtained by examining the contents of the processor

means 132 as a function of location of the confocal region 118. This image may be used to

identify zones of interest 134, such as subcutaneous tumors or other diseased areas.

10   SECOND EXEMPLARY EMBODIMENT OF THE INVENTION:

       As an alternate to this preferred embodiment, a modulation means may be incorporated into

the general embodiment shown in FIGURE 11: such modulation means may be used to improve

overall performance of the imaging system, such as to improve rejection of environmental or

instrumental noise sources, to enable recovery of pure two-photon excited emission at the second

15   harmonic, or to facilitate detection of emitted light using phase photometric approaches.

Specifically, FIGURE 12 shows that a modulator means 136, such as an electro-optic or acousto-

optic modulator, a chopper, or other means, located so as to interact with the beam of NIR

radiation 110 emitted by the NIR source 108 can be used to encode the beam of NIR radiation 110

with a modulation pattern that is registered to the output of a modulator driver 138 that provides a

20   drive signal 140 to the modulation means 136. The modulated beam of NIR radiation 142 thereby

produced is then directed onto the specimen 116 as described previously for FIGURE 11. The

two-photon excited emitted light 144 thereby produced will exit the confocal region 118 in an

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essentially isotropic manner. However, in contrast to the similar emitted light 122 described

previously for FIGURE 11, this emitted light 144 will exhibit a modulation that is essentially

synchronous with the modulation of the modulated beam of NIR radiation 142, which in turn is

synchronous with the drive signal 140 issued by the modulator driver 138. A portion of the

5   modulated emitted light 146 is captured by a detection means 126, such as a photomultiplier tube,

that is mounted at a position inside or outside of the specimen 116. This detection means 126 is

fitted with a wavelength selection means 128, such as an optical bandpass filter, that serves to

process the captured portion of the modulated emitted light 146 in such a way that the selection

means 128 rejects a major fraction of the elastically scattered light while passing a major fraction of

10   light at the wavelength or wavelengths corresponding to that which is principally characteristic of

emission from the diagnostic agent. The modulated signal thus issued 148 from the detection

means 126 is captured by a processor means 150. The processor means 150 serves two primary

purposes, firstly to demodulate the modulated signal thus issued 148 from the detection means 126

using a demodulation reference output 152 issued by the modulator driver 138, and secondly to

15   record demodulated emission response from the diagnostic imaging agent as a function of location

of the confocal region 118. Hence, by causing the location of the confocal region 118 to be

scanned throughout the volume of the specimen 116, a complete image of the specimen 116 may

be obtained by examining the contents of the processor means 150 as a function of location of the

confocal region 118. This image may be used to identify zones of interest 134, such as

20   subcutaneous tumors or other diseased areas.

THIRD EXEMPLARY EMBODIMENT OF THE INVENTION:

As a second alternate to this preferred embodiment, an unfocused beam of NIR radiation

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may be used to illuminate superficial features of a specimen to provide a direct imaging means of detection. This is shown in FIGURE 13. Specifically, the output of a NIR source, such as the mode-locked titanium:sapphire laser, can be used to induce simultaneous two-photon photo-activation of endogenous or exogenous diagnostic imaging agents present on or near the surface of

5   a specimen using light at a wavelength approximately twice that necessary for conventional single-photon photo-activation. The NIR Source 108 produces a beam of NIR radiation 110 consisting of a rapid series of high peak power pulses of NIR radiation. This beam is modulated using a modulator means 136 located so as to interact with the beam of NIR radiation 110 emitted by the NIR source 108. This modulator means 136 encodes the beam of NIR radiation 110 with a

10   modulation pattern that is registered to the output of a modulator driver 138 that provides a drive signal 140 to the modulation means 136. The modulated beam of NIR radiation 142 thereby produced is then defocused using standard optical means, such as reflective or refractive optics 154, to produce a divergent excitation beam 156 that is directed onto a specimen 116 to be imaged. Simultaneous two-photon photo-activation of diagnostic imaging agent present on or near the

15   surface of the specimen 116 produces modulated two-photon excited emitted light 144 having a modulation that is essentially synchronous with the modulation of the modulated beam of NIR radiation 142, which in turn is synchronous with the drive signal 140 issued by the modulator driver 138. A portion of the modulated emitted light 146 is captured by an imaging detection means 158, such as a charge coupled device array, that is mounted at a position outside of the

20   specimen 116. This imaging detection means 158 is fitted with a wavelength selection means 128, such as an optical bandpass filter, that serves to process the captured portion of the modulated emitted light 146 in such a way that the selection means 128 rejects a major fraction of the

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elastically scattered light while passing a major fraction of light at the wavelength or wavelengths corresponding to that which is principally characteristic of emission from the diagnostic agent. The modulated signal thus issued 160 from the imaging detection means 158 is captured by a processor means 162. The processor means 162 serves two primary purposes, firstly to demodulate the

5   modulated signal thus issued 160 from the imaging detection means 158 using a demodulation reference output 152 issued by the modulator driver 138. and secondly to record demodulated emission response from the diagnostic imaging agent as a function of location of emission. Hence, this alternate embodiment enables direct videographic imaging of surface features 164, such as skin cancer lesions, to be performed based on spatial differences in two-photon excited emission across

10   the illuminated surface of the specimen 116.

It will be understood that each of the elements described above, or two or more together, may also find useful application in other types of constructions or applications differing from the types described above.

While the invention has been illustrated and described as embodied in a general method for

15   improved selectivity in photo-activation of molecular diagnostic imaging agents, it is not intended to be limited to the details shown, since it will be understood that various omissions, modifications, substitutions and changes in the forms and details of the method illustrated and in its operation can be made by those skilled in the art without departing in any way from the spirit of the present invention. For example, in the third exemplary embodiment, the modulation and

20   demodulation details may be omitted to produce a more simple imaging apparatus, although this example modification would yield an overall reduction in imaging performance.

Without further analysis, the foregoing will so fully reveal the gist of the present invention

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that others can, by applying current knowledge, readily adapt it for various applications without omitting features that, from the standpoint of prior art, fairly constitute essential characteristics of the generic or specific aspects of this invention.

What is claimed as new and desired to be protected by Letters Patent is set forth in the

5   appended claims.

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WHAT IS CLAIMED IS:

Claim 1.   A method for the imaging of a particular volume of plant or animal tissue, wherein the plant or animal tissue contains at least one photo-active molecular agent. the method comprising the steps of:

(a)   treating the particular volume of the plant or animal tissue with light sufficient to promote a simultaneous two-photon excitation of the photo-active molecular agent contained in the particular volume of the plant or animal tissue;

(b)   photo-activating at least one of the at least one photo-active molecular agent in the particular volume of the plant or animal tissue, thereby producing at least one photo-activated molecular agent, wherein the at least one photo-activated molecular agent emits energy;

(c)   detecting the energy emitted by the at least one photo-activated molecular agent; and

(d)   producing a detected energy signal which is characteristic of the particular volume of plant or animal tissue.

Claim 2.   The method of Claim 1 wherein the light sufficient to promote a simultaneous two-photon excitation of the at least one photo-active molecular agent is laser light.

Claim 3.   The method of Claim 1 wherein the light sufficient to promote a simultaneous two-photon excitation of the photo-active molecular agent is a focused beam of light.

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Claim 4.   The method of Claim 4 wherein the focused beam of light is focused laser light.

Claim 5.   The method of Claim 1 further including a first step of treating the plant or animal tissue with at least one photo-active molecular agent, wherein the particular volume of the plant or animal tissue retains at least a portion of the at least one photo-active molecular agent.

Claim 6.   The method of Claim 5 wherein the at least one photo-active molecular agent is selected from the group consisting of psoralen, 5-methoxypsoralen (5-MOP), 8-methoxypsoralen (8-MOP), 4,5',8-trimethylpsoralen (TMP), 4'-aminomethyl-4,5',8-trimethylpsoralen (AMT), 5-chloromethyl-8-methoxypsoralen (HMT), angelicin (isopsoralen), 5-methylangelicin (5-MIP), 3-carboxypsoralen, porphyrin, haematoporphyrin derivative (HPD), photofrin II, benzoporphyrin derivative (BPD), protoporphyrin IX (PpIX), dye haematoporphyrin ether (DHE), polyhaematoporphyrin esters (PHE), 13,17-N,N,N-dimethylethylethanolamine ester of protoporphyrin (PH1008), tetra(3-hydroxyphenyl)-porphyrin (3-THPP), tetraphenylporphyrin monosulfonate (TPPS1), tetraphenylporphyrin disulfonate (TPPS2a), dihaematoporphyrin ether, mesotetraphenylporphyrin, mesotetra(4N-methylpyridyl)porphyrin (T4MpyP), octa-(4-*tert*-butylphenyl)tetrapyrazinoporphyrazine (OPTP), phthalocyanine, tetra-(4-*tert*-butyl)phthalocyanine (t_4-PcH$_2$), tetra-(4-*tert*-butyl)phthalocyanatomagnesium (t_4-PcMg), chloroaluminum sulfonated phthalocyanine (CASPc), chloroaluminum phthalocyanine tetrasulfate (AlPcTS), mono-sulfonated aluminum phthalocyanine (AlSPc), di-sulfonated aluminum phthalocyanine (AlS2Pc), tri-sulfonated aluminum phthalocyanine (AlS3Pc), tetra-sulfonated aluminum phthalocyanine

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(AlS4Pc), silicon phthalocyanine (SiPc IV), zinc II phthalocyanine (ZnPc), bis(di-isobutyl

octadecylsiloxy)silicon 2,3-naphthalocyanine (isoBOSINC), germanium IV

octabutoxyphthalocyanine (GePc), rhodamine 101 (Rh-101), rhodamine 110 (Rh-110), rhodamine

123 (Rh-123), rhodamine 19 (Rh-19), rhodamine 560 (Rh-560), rhodamine 575 (Rh-575),

20   rhodamine 590 (Rh-590), rhodamine 610 (Rh-610), rhodamine 640 (Rh-640), rhodamine 6G (Rh-

6G), rhodamine 700 (Rh-700), rhodamine 800 (Rh-800), rhodamine B (Rh-B), sulforhodamine

101, sulforhodamine 640, sulforhodamine B, coumarin 1, coumarin 2, coumarin 4, coumarin 6,

coumarin 6H, coumarin 7, coumarin 30, coumarin 47, coumarin 102, coumarin 106, coumarin

120, coumarin 151, coumarin 152, coumarin 152A, coumarin 153, coumarin 311, coumarin 307,

25   coumarin 314, coumarin 334, coumarin 337, coumarin 343, coumarin 440, coumarin 450,

coumarin 456, coumarin 460, coumarin 461, coumarin 466, coumarin 478, coumarin 480,

coumarin 481, coumarin 485, coumarin 490, coumarin 500, coumarin 503, coumarin 504,

coumarin 510, coumarin 515, coumarin 519, coumarin 521, coumarin 522, coumarin 523,

coumarin 535, coumarin 540, coumarin 540A, coumarin 548, 5-ethylamino-9-

30   diethylaminobenzo[a]phenoxazinium (EtNBA), 5-ethyl-amino-9-diethyl-

aminobenzo[a]phenothiazinium (EtNBS), 5-ethylamino-9-diethylaminobenzo[a]pheno-

selenazinium (EtNBSe), chlorpromazine, chlorpromazine derivatives, chlorophyll derivatives,

bacteriochlorophyll derivatives, metal-ligand complexes, tris(2,2'-bipyridine)ruthenium (II)

dichloride (RuBPY), tris(2,2'-bipyridine)rhodium (II) dichloride (RhBPY), tris(2.2'-

35   bipyridine)platinum (II) dichloride (PtBPY), pheophorbide a, merocyanine 540, vitamin D, 5-

amino-laevulinic acid, photosan, chlorin e6, chlorin e6 ethylenediamide, mono-L-aspartyl chlorin

e6, phenoxazine Nile blue derivatives, stilbene, stilbene derivatives, 4-(N-(2-hydroxyethyl)-N-

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methyl)-aminophenyl)-4'-(6-hydroxyhexylsulfonyl)stilbene (APSS), and standard biological dyes and stains.

Claim 7.   The method of Claim 5 wherein the at least one photo-active molecular agent is at least one biogenic photo-active molecular agent that is specific to a particular tissue within the particular volume of plant or animal tissue.

Claim 8.   The method of Claim 7 wherein the at least one biogenic photo-active molecular agent includes a segment selected from the group consisting of DNA, RNA, amino acids, proteins, antibodies, ligands, haptens, carbohydrate receptors or complexing agents, lipid receptors or complexing agents, protein receptors or complexing agents, chelators, and encapsulating vehicles.

Claim 9.   The method of Claim 8 wherein the at least one biogenic photo-active molecular agent further includes a segment which is photo-activated when subject to light sufficient to promote a simultaneous two-photon excitation.

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Claim 10.   The method of Claim 1 wherein the step of treating the particular volume of the plant or animal tissue with light sufficient to promote a simultaneous two-photon excitation of the at least one photo-active molecular agent contained in the particular volume of the plant or

5   animal tissue includes the steps of:

(a1)   modulating light from a light source with a particular type of modulation , thereby producing a modulated light; and

(a2)   treating the particular volume of the plant or animal tissue with the modulated light sufficient to promote a simultaneous two-photon excitation of the at least one photo-active

10   molecular agent contained in the particular volume of the plant or animal tissue;

and further including the steps of:

(e)   demodulating the dectected energy signal with the particular type of modulation; and

(f)   producing a demodulated energy signal which is characteristic of the particular volume of the plant or animal tissue.

Claim 11.   The method of Claim 10 wherein the step of demodulating the dectected energy signal with the particular type of modulation includes demodulating the detected energy signal at a frequency twice that of the particular type of modulation, thereby detecting the second harmonic of the particular type of modulation.

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Claim 12.   The method of Claim 10 wherein the demodulated energy signal which is characteristic of the particular volume of the plant or animal tissue represents a change in lifetime of at least one photo-activated molecular agent present in the particular volume of the plant or animal

5   tissue.

Claim 13.   A method for the imaging of a particular volume of material, wherein the material contains at least one photo-active molecular agent, the method comprising the steps of:

(a)   treating the particular volume of the material with light sufficient to promote a simultaneous two-photon excitation of at least one of the at least one photo-active molecular agent contained in the particular volume of the material;

(b)   photo-activating the at least one photo-active molecular agent in the particular volume of the material, thereby producing at least one photo-activated molecular agent, wherein the at least one photo-activated molecular agent emits energy;

(c)   detecting the energy emitted by the at least one photo-activated molecular agent; and

(d)   producing a detected energy signal which is characteristic of the particular volume of the material.

Claim 14.   The method of Claim 13 wherein the material is selected from the group consisting of plant tissue and animal tissue.

Claim 15.   The method of Claim 13 wherein the light sufficient to promote a simultaneous two-photon excitation of the at least one photo-active molecular agent is laser light.

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Claim 16.   The method of Claim 13 wherein the light sufficient to promote a simultaneous two-photon excitation of the at least one photo-active molecular agent is a focused beam of light.

Claim 17.   The method of Claim 16 wherein the focused beam of light is focused laser light.

Claim 18.   The method of Claim 13 further including a first step of treating the material with at least one photo-active molecular agent, wherein the particular volume of the material retains at least a portion of the at least one photo-active molecular agent.

Claim 19.   The method of Claim 18 wherein the at least one photo-active molecular agent is selected from the group consisting of psoralen, 5-methoxypsoralen (5-MOP), 8-methoxypsoralen (8-MOP), 4,5',8-trimethylpsoralen (TMP), 4'-aminomethyl-4,5',8-trimethylpsoralen (AMT), 5-chloromethyl-8-methoxypsoralen (HMT), angelicin (isopsoralen), 5-methylangelicin (5-MIP), 3-carboxypsoralen, porphyrin, haematoporphyrin derivative (HPD), photofrin II, benzoporphyrin derivative (BPD), protoporphyrin IX (PpIX), dye haematoporphyrin ether (DHE), polyhaematoporphyrin esters (PHE), 13,17-N,N,N-dimethylethylethanolamine ester of protoporphyrin (PH1008), tetra(3-hydroxyphenyl)-porphyrin (3-THPP), tetraphenylporphyrin monosulfonate (TPPS1), tetraphenylporphyrin disulfonate (TPPS2a), dihaematoporphyrin ether, mesotetraphenylporphyrin, mesotetra(4N-methylpyridyl)porphyrin (T4MpyP), octa-(4-tert-

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(t_4-PcH$_2$), tetra-(4-*tert*-butyl)phthalocyanatomagnesium (t_4-PcMg), chloroaluminum sulfonated phthalocyanine (CASPc), chloroaluminum phthalocyanine tetrasulfate (AlPcTS), mono-sulfonated aluminum phthalocyanine (AlSPc), di-sulfonated aluminum phthalocyanine (AlS2Pc), tri-

15 sulfonated aluminum phthalocyanine (AlS3Pc), tetra-sulfonated aluminum phthalocyanine (AlS4Pc), silicon phthalocyanine (SiPc IV), zinc II phthalocyanine (ZnPc), bis(di-isobutyl octadecylsiloxy)silicon 2,3-naphthalocyanine (isoBOSINC), germanium IV octabutoxyphthalocyanine (GePc), rhodamine 101 (Rh-101), rhodamine 110 (Rh-110), rhodamine 123 (Rh-123), rhodamine 19 (Rh-19), rhodamine 560 (Rh-560), rhodamine 575 (Rh-575),

20 rhodamine 590 (Rh-590), rhodamine 610 (Rh-610), rhodamine 640 (Rh-640), rhodamine 6G (Rh-6G), rhodamine 700 (Rh-700), rhodamine 800 (Rh-800), rhodamine B (Rh-B), sulforhodamine 101, sulforhodamine 640, sulforhodamine B, coumarin 1, coumarin 2, coumarin 4, coumarin 6, coumarin 6H, coumarin 7, coumarin 30, coumarin 47, coumarin 102, coumarin 106, coumarin 120, coumarin 151, coumarin 152, coumarin 152A, coumarin 153, coumarin 311, coumarin 307,

25 coumarin 314, coumarin 334, coumarin 337, coumarin 343, coumarin 440, coumarin 450, coumarin 456, coumarin 460, coumarin 461, coumarin 466, coumarin 478, coumarin 480, coumarin 481, coumarin 485, coumarin 490, coumarin 500, coumarin 503, coumarin 504, coumarin 510, coumarin 515, coumarin 519, coumarin 521, coumarin 522, coumarin 523, coumarin 535, coumarin 540, coumarin 540A, coumarin 548, 5-ethylamino-9-

30 diethylaminobenzo[a]phenoxazinium (EtNBA), 5-ethyl-amino-9-diethyl-aminobenzo[a]phenothiazinium (EtNBS), 5-ethylamino-9-diethylaminobenzo[a]pheno-selenazinium (EtNBSe), chlorpromazine, chlorpromazine derivatives, chlorophyll derivatives, bacteriochlorophyll derivatives, metal-ligand complexes, tris(2,2'-bipyridine)ruthenium (II)

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bacteriochlorophyll derivatives, metal-ligand complexes, tris(2,2'-bipyridine)ruthenium (II)

dichloride (RuBPY), tris(2,2'-bipyridine)rhodium (II) dichloride (RhBPY), tris(2,2'-

35   bipyridine)platinum (II) dichloride (PtBPY), pheophorbide a, merocyanine 540, vitamin D, 5-

amino-laevulinic acid, photosan, chlorin e6, chlorin e6 ethylenediamide, mono-L-aspartyl chlorin

e6, phenoxazine Nile blue derivatives, stilbene, stilbene derivatives, 4-(N-(2-hydroxyethyl)-N-

methyl)-aminophenyl)-4'-(6-hydroxyhexylsulfonyl)stilbene (APSS), and standard biological dyes

and stains.

Claim 20.   The method of Claim 18 wherein the at least one photo-active molecular

agent is at least one biogenic photo-active molecular agent that is specific to a particular substance

within the particular volume of material.

Claim 21.   The method of Claim 20 wherein the at least one biogenic photo-active molecular

agent includes a segment selected from the group consisting of DNA, RNA, amino acids, proteins,

antibodies, ligands, haptens, carbohydrate receptors or complexing agents, lipid receptors or complexing

agents, protein receptors or complexing agents, chelators, and encapsulating vehicles.

Claim 22.   The method of Claim 21 wherein the at least one biogenic photo-active molecular

agent further includes a segment which is photo-activated when subject to light sufficient to promote a

simultaneous two-photon excitation.

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Claim 23.   The method of Claim 13 wherein the step of treating the particular volume of the

material with light sufficient to promote a simultaneous two-photon excitation of the at least one photo-

active molecular agent contained in the particular volume of the material includes the steps of:

5   (a1)   modulating light from a light source with a particular type of modulation , thereby

producing a modulated light; and

(a2)   treating the particular volume of the material with the modulated light sufficient to promote

a simultaneous two-photon excitation of the at least one photo-active molecular agent contained in

the particular volume of the material;

10   and further including the steps of:

(e)   demodulating the dectected energy signal with the particular type of modulation; and

(f)   producing a demodulated energy signal which is characteristic of the particular volume of

the material.

Claim 24.   The method of Claim 23 wherein the step of demodulating the dectected energy

signal with the particular type of modulation includes demodulating the detected energy signal at a

frequency twice that of the particular type of modulation, thereby detecting the second harmonic of the

particular type of modulation.

Claim 25.   The method of Claim 23 wherein the demodulated energy signal which is

characteristic of the particular volume of the material represents a change in lifetime of at least one photo-

activated molecular agent present in the particular volume of the material.

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METHOD FOR IMPROVED SELECTIVITY IN PHOTO-ACTIVATION AND DETECTION OF MOLECULAR DIAGNOSTIC AGENTS

ABSTRACT OF THE INVENTION

A method for the imaging of a particular volume of plant or animal tissue, wherein the plant or animal tissue contains at least one photo-active molecular agent. The method comprises the steps of treating the particular volume of the plant or animal tissue with light sufficient to promote a simultaneous two-photon excitation of the photo-active molecular agent contained in the particular volume of the plant or

5   animal tissue, photo-activating at least one of the at least one photo-active molecular agent in the particular volume of the plant or animal tissue, thereby producing at least one photo-activated molecular agent, wherein the at least one photo-activated molecular agent emits energy, detecting the energy emitted by the at least one photo-activated molecular agent, and producing a detected energy signal which is characteristic of the particular volume of plant or animal tissue. The present invention also provides a method for the

10   imaging of a particular volume of material, wherein the material contains at least one photo-active molecular agent.

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and Detection of Molecular Diagnostic Agents
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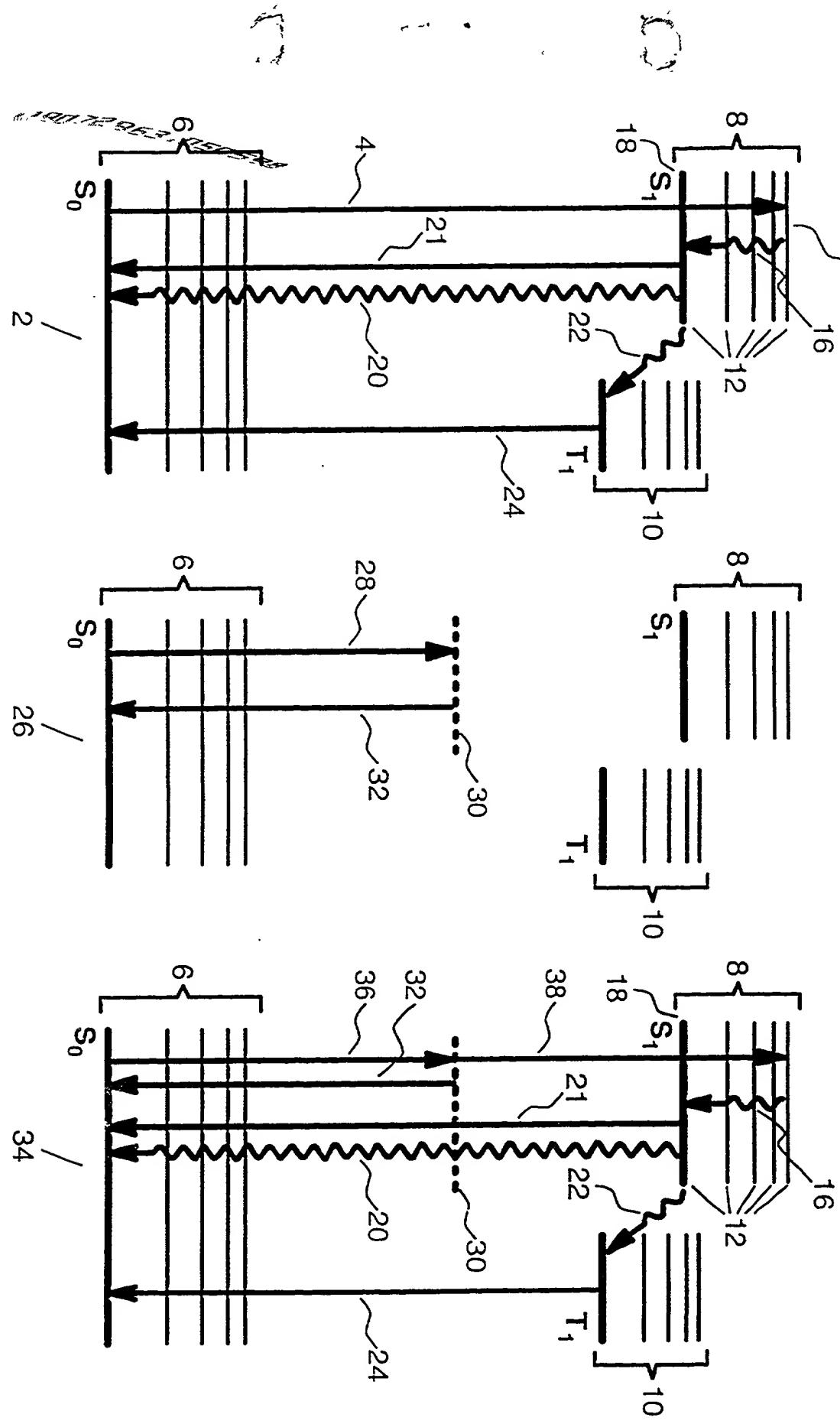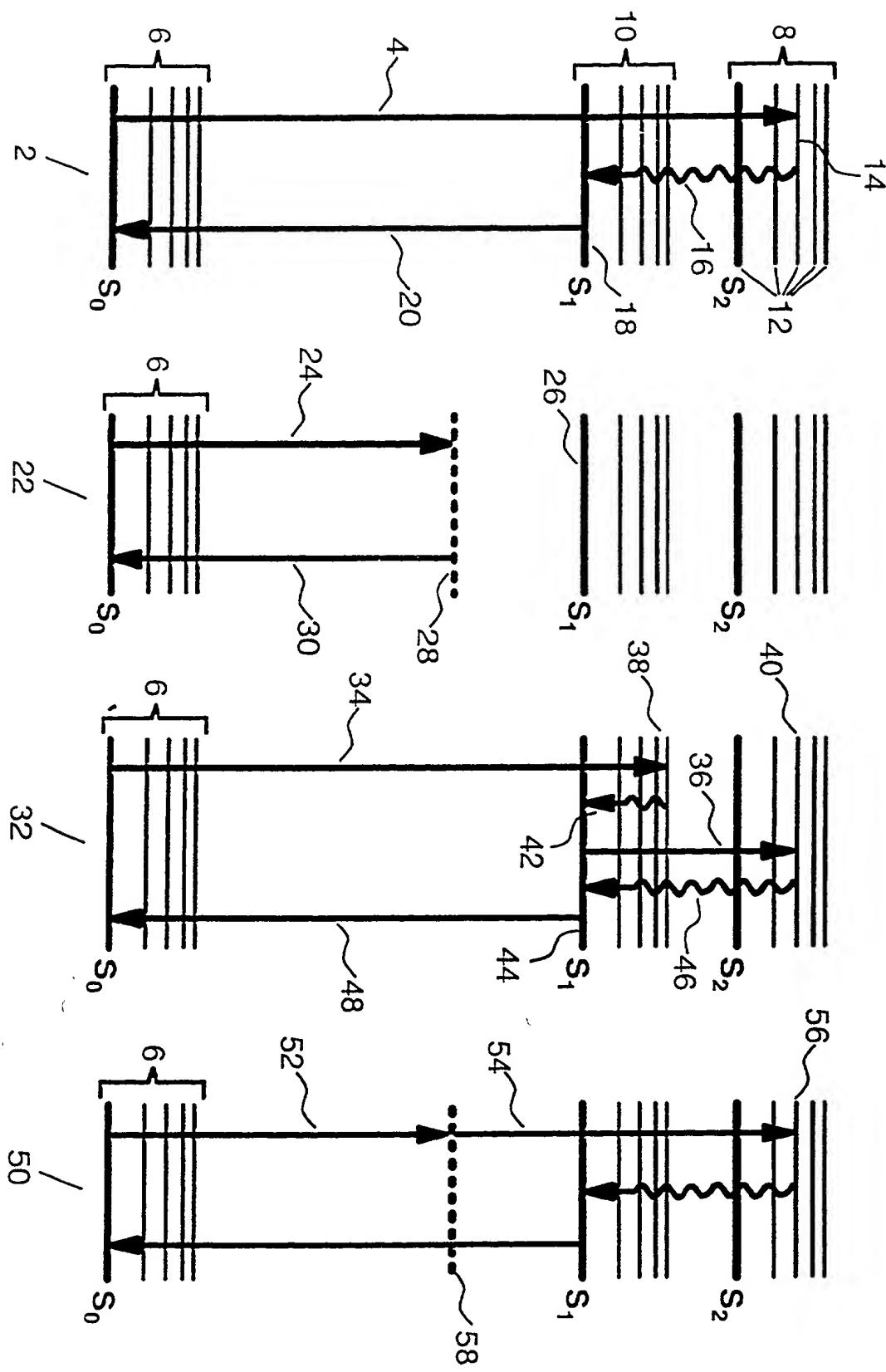
Figure 1

Figure 1

Figure 2

Figure 3

Figure 4

Relative Scatter

Wavelength (nm)

66

68

70

72
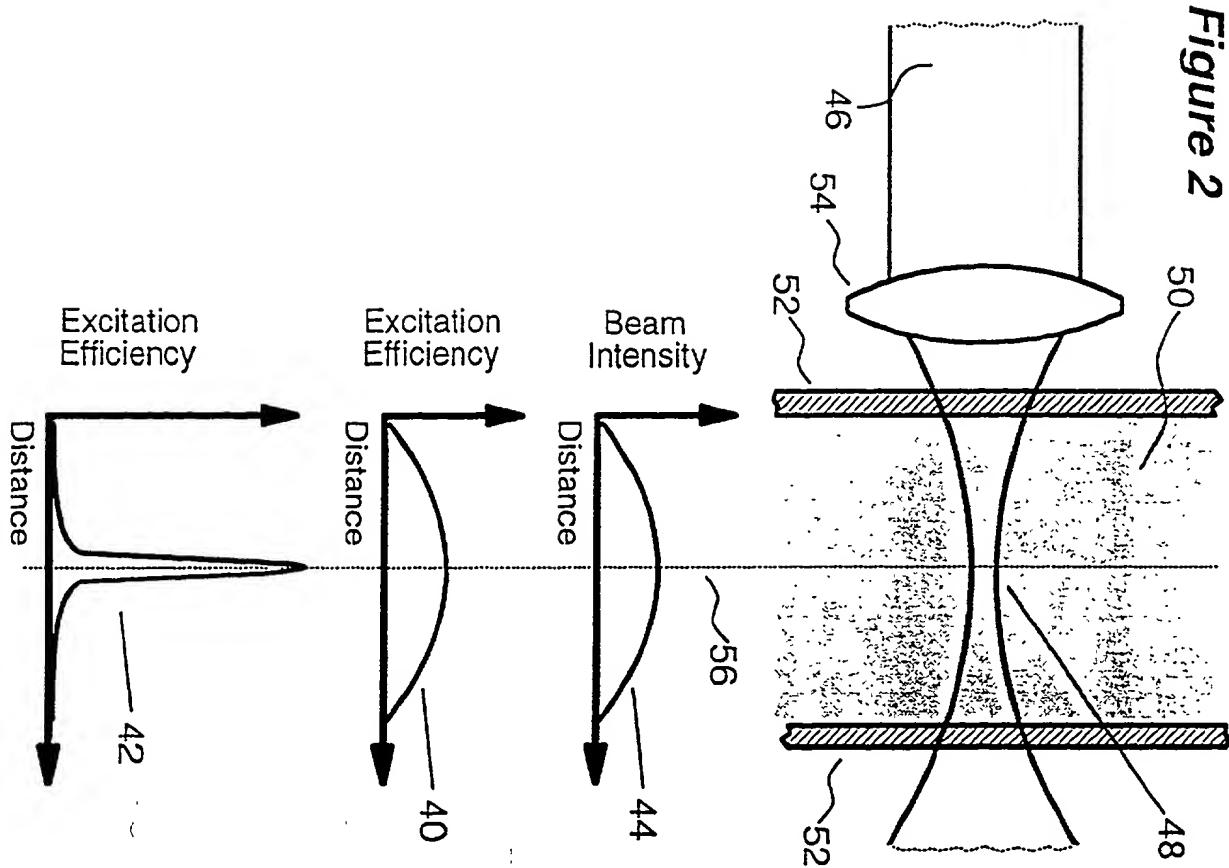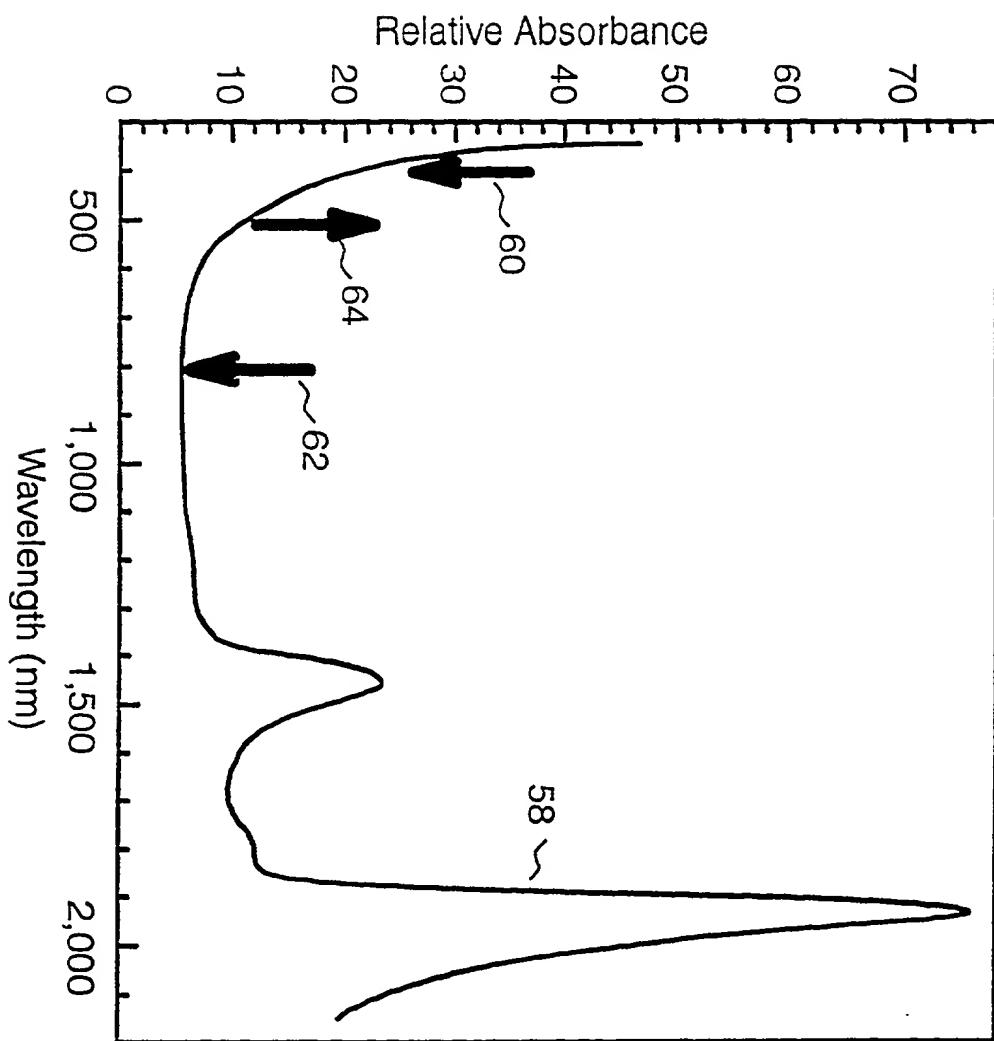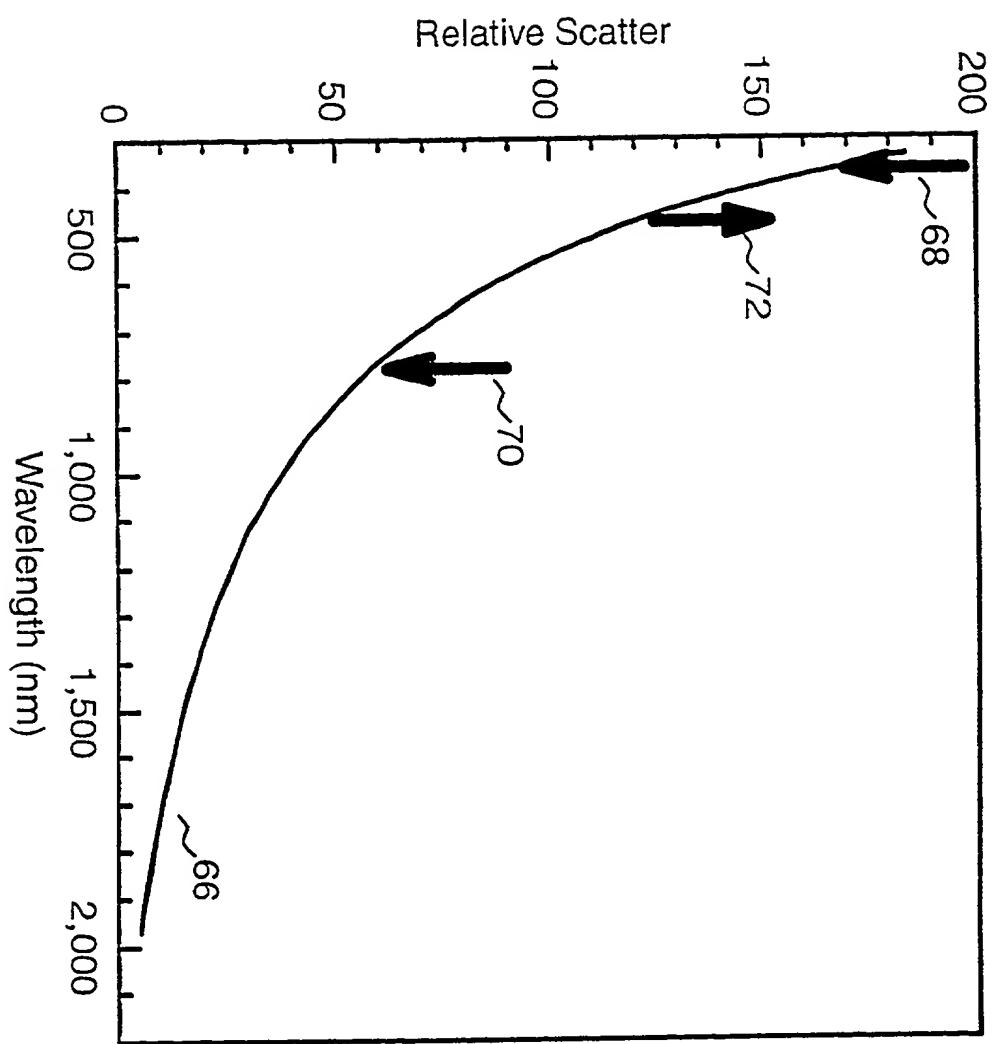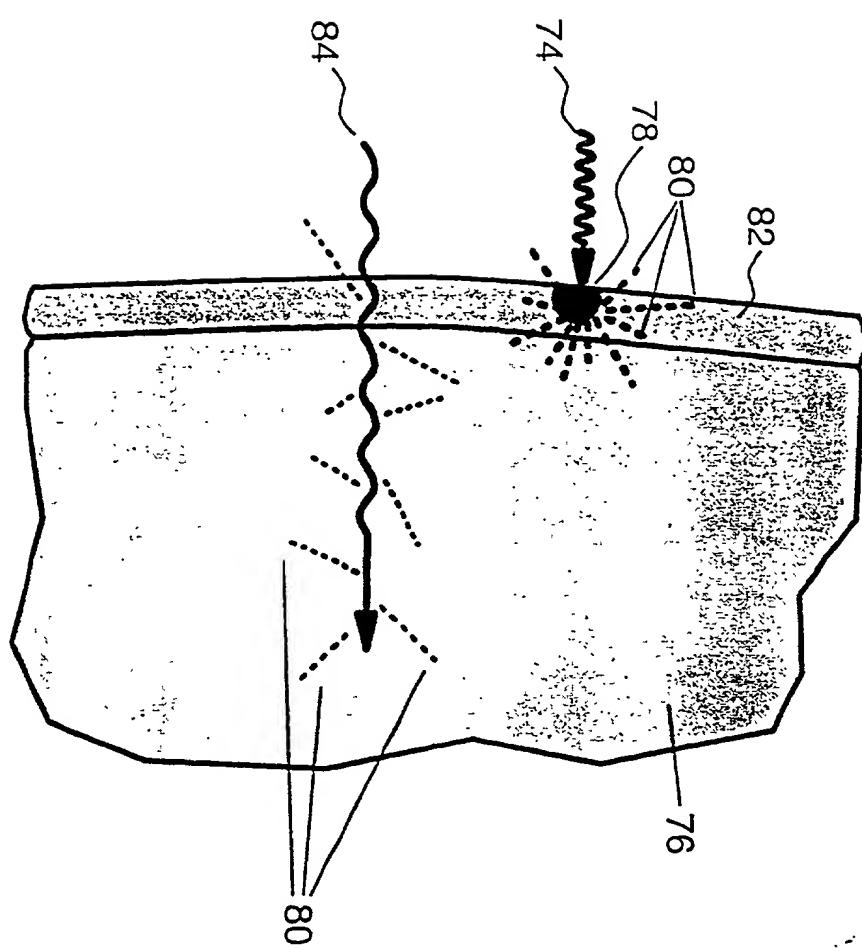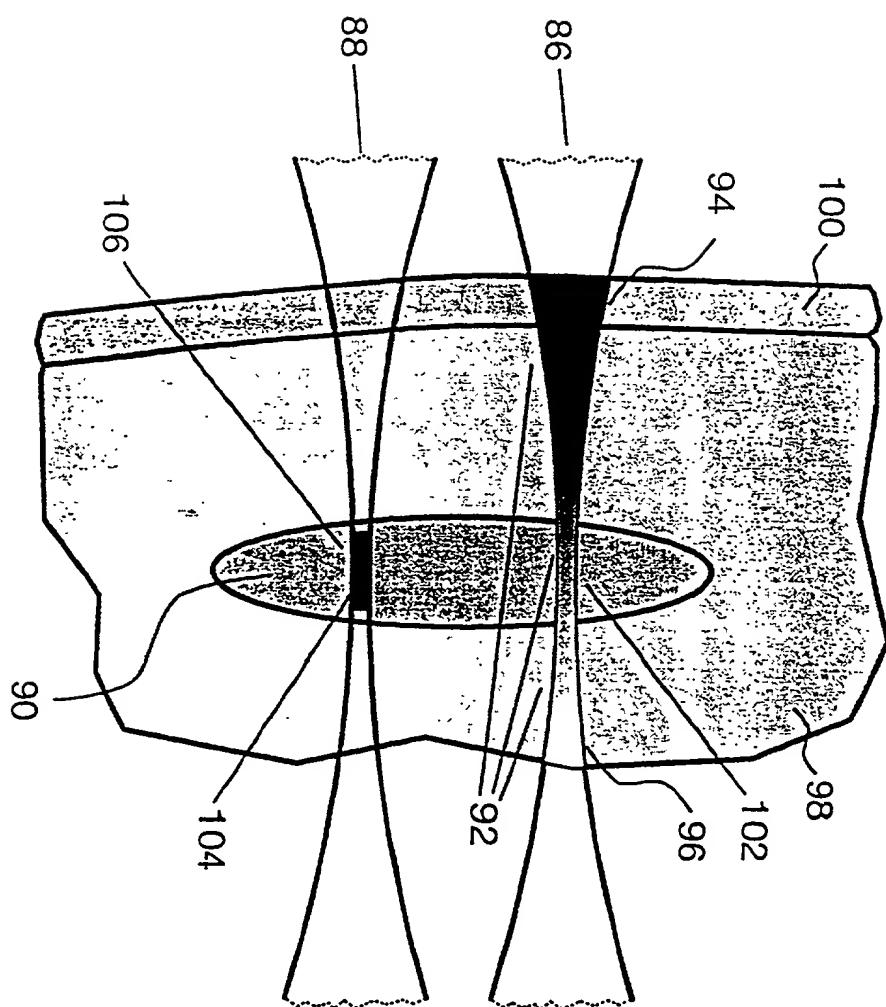
Figure 5

Figure 6

Figure 11
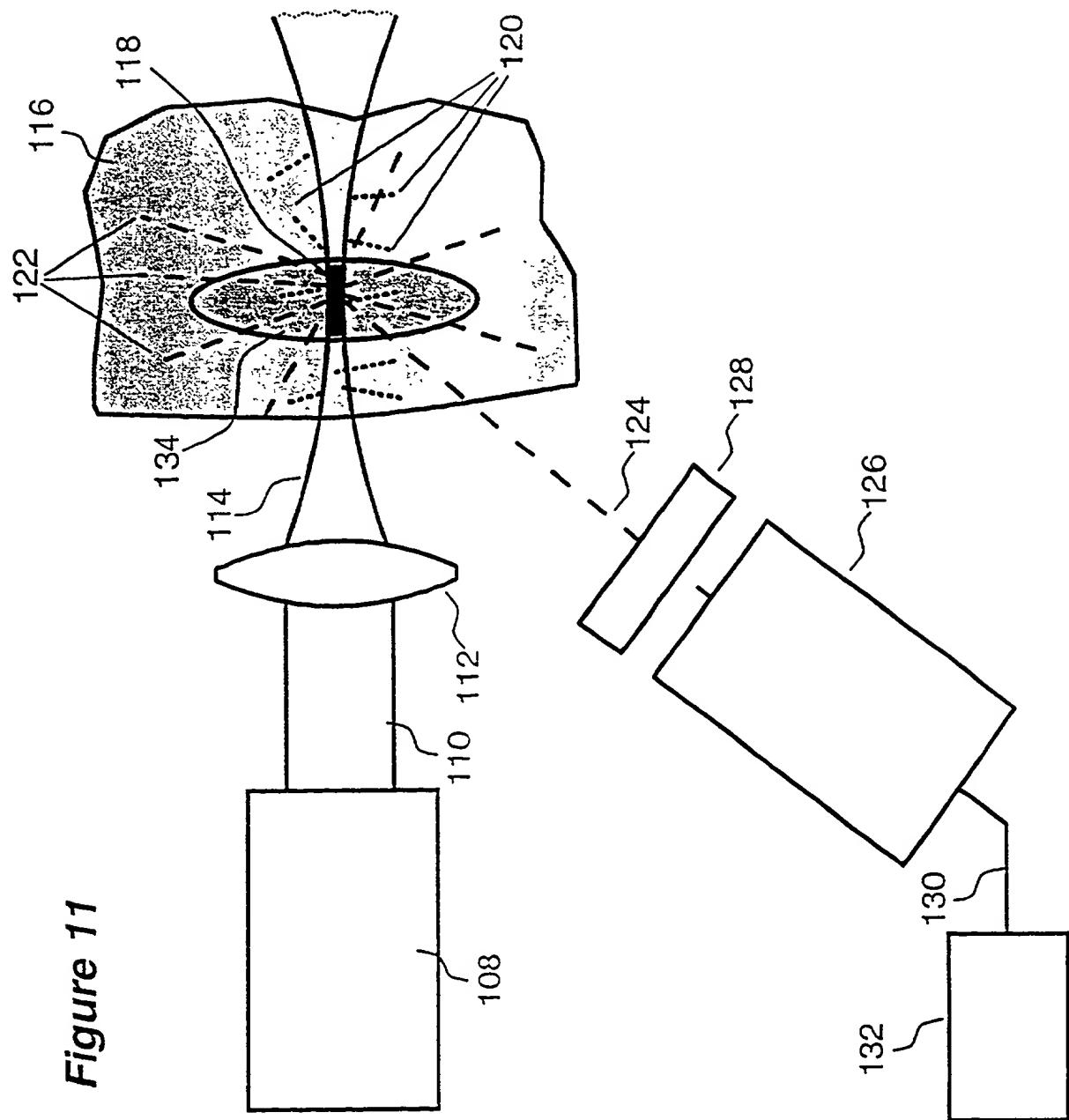
Figure 12

Figure 13

<u>IN THE UNITED STATES PATENT AND TRADEMARK OFFICE</u>

In re Application of:)

    Eric Wachter et al.   )

Serial No.:   )

Filed: Herewith   )

For: Method For Improved Selectivity)
         In Photoactivation And   )
         Detection Of Molecular   )
         Diagnostic Agents   )

Examiner:)

Art Unit:)

May 5, 1998

<u>**ASSIGNEE'S STATEMENT OF STATUS**</u>
<u>**AS A SMALL ENTITY**</u>

Assistant Commissioner for Patents
Washington, D.C. 20231

Dear Sir:

The undersigned Declarant, on behalf of the assignee of the captioned application, respectfully submit this verified STATEMENT OF STATUS AS A SMALL ENTITY pursuant to 37 C.F.R. §1.9 and 1.27.

1.   PHOTOGEN, INC. a Tennessee corporation existing under the State of Tennessee is the Assignee of the entire right, title and interest in the above-identified patent application of Eric Wachter and H. Craig Dees filed herewith as shown by the Assignment, a copy of which is attached hereto.

2.   Declarant is the Secretary of PHOTOGEN, INC. a corporation organized and existing under the laws of the State of

Tennessee, having offices at 7327 Oak Ridge Highway, Knoxville, Tennessee 37931. Declarant is empowered to act on behalf of PHOTOGEN, INC. for matters relating to prosecution of the above-identified patent application.

3.   PHOTOGEN, INC. has not assigned, granted, conveyed, or licensed, and is under no obligation under contract or law to assign, grant, convey or license any rights in the invention or application to any person.

4.   PHOTOGEN, INC. is a small business concern as defined by 37 C.F.R. §1.9(d), having fewer than 500 employees.

Declarant acknowledges PHOTOGEN'S obligation to notify the United States Patent Office, prior to or at the time of paying the issue fee in this case, of any change in its status as a small business concern, or of any obligation to assign, grant, convey or license or any actual assignment, grant, conveyance, or license of the above cited patent application to other than a small entity as defined by 37 C.F.R. §1.9.

Declarant acknowledges that willful false statements and the like so made herein are punishable by fine or imprisonment, or both, under 18 U.S.C. §1001, and that such willful false statements may jeopardize the validity of any patent resulting therefrom.

PHOTOGEN, INC.

Dated: 5 May 98   BY: _____

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of:)
)
    Eric Wachter et al.)
)
Serial No.: 08/741,370)
)
Filed: October 30, 1996)
)
For: Method For Improved Selectivity)
          In Photoactivation And)
          Detection Of Molecular)
          Diagnostic Agents)
)
Examiner: K. Riskin)
)
Art Unit: 3312)

I hereby certify that this correspondence is being deposited with the United States Postal Service as first class mail in an envelope addressed to. Assistant Commissioner for Patents, Washington, D.C. 20231, on *March 16, 1998*

Sandra Sotto

Date _*March 16, 1998*_

REVOCATION OF POWER OF PRIOR POWER OF ATTORNEY
AND APPOINTMENT OF NEW POWER OF ATTORNEY

Please revoke all prior powers of attorney.

I hereby appoint the following attorney(s) and/or agent(s) to prosecute this application and transact all business in the Patent and Trademark Office connected therewith.

Edward D. Manzo, 28,139

Send correspondence to:

Edward D. Manzo, Esq.
COOK, MCFARRON & MANZO, LTD.
135 South LaSalle Street
Suite 4100
Chicago, Illinois 60603

Direct telephone calls to:

Edward D. Manzo, Esq.
  (312) 236-8500

I am an officer of the assignee of record of the entire interest and authorized to sign on behalf of the assignee.

Eric Wachter
Secretary
Photogen Inc.

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of:	)
	)
Eric Wachter et al.	)
	)
Serial No.:	)
	)
Filed: Herewith	)
	)
For: Method For Improved Selectivity	)
In Photoactivation And	)
Detection Of Molecular	)
Diagnostic Agents	)
	)

"Express Mail" Mailing Label No. _EI0239 232~~79~~ 945~~

Date of Deposit _MAY 5, 1998_

I hereby certify that this paper or fee is being deposited with the United States Postal Service "Express Mail Post Office to Addressee" service under 37 CFR 1.10 on the date indicated above and is addressed to the Assistant Commissioner for Patents, Washington, D.C. 20231.

Name _Casindra Himmelstein_
(typed or printed)

Signature _[signature]_

May 5, 1998

Assistant Commissioner for Patents
Washington, D.C. 20231

APPOINTMENT OF ASSOCIATED ATTORNEYS

Sir:

Please recognize the following as my associate attorneys in the above-captioned application:

Granger Cook, Jr.	Reg. No. 18,283
Gary W. McFarron	Reg. No. 27,357
Stephen B. Heller	Reg. No. 30,181
Andrew G. Kolomayets	Reg. No. 33,723
Mark J. Murphy	Reg. No. 34,225
John R. Lagowski	Reg. no. P41,922

Respectfully submitted,

[signature] Edward Manzo
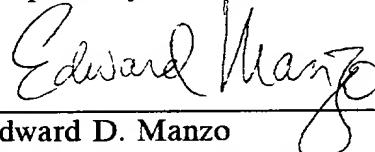
Edward D. Manzo
Registration no: 28,139

Dated: May 5, 1998

COOK, McFARRON & MANZO, LTD.
200 W. Adams Street
Suite 2850
Chicago, Illinois 60606
(312) 236-8500

DECLARATION AND POWER OF ATTORNEY

As a below named inventors, we hereby declare that:

Our residences, post office addresses and citizenships are as stated below next to our names.

We believe we are an original, first and joint inventors of the subject matter which is claimed and for which a patent is sought on the invention entitled <u>METHOD FOR IMPROVED SELECTIVITY IN PHOTO-ACTIVATION AND DETECTION OF MOLECULAR DIAG-NOSTIC AGENTS</u>,the specification of which

(check [X]   is attached hereto
one)   [ ]   was filed on _____ as Application Serial No. _____ and was amended on _____.
(if applicable)

We hereby state that we have reviewed and understand the contents of the above-identified specification, including the claims, as amended by any amendment referred to above.

We acknowledge the duty to disclose information which is material to the patentability of this application in accordance with 37 C.F.R. § 1.56.

We hereby claim foreign priority benefits under 35 U.S.C. § 119 of any foreign application(s) for patent or inventor's certificate listed below and have also identified below any foreign application for patent or inventor's certificate having a filing date before that of the application on which priority is claimed:
Prior Foreign Application(s)

Priority Claimed

(Number)	(Country)	(Day/Month/Year Filed)	[ ] Yes	[ ] No
(Number)	(Country)	(Day/Month/Year Filed)	[ ] Yes	[ ] No

We hereby claim the benefit under 35 U.S.C. § 120 of any United States application(s) listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States application in the manner provided by the first paragraph of 35 U.S.C. § 112, acknowledge the duty to disclose material information as defined in 37 C.F.R. § 1.56 which occurred between the filing date of the prior application and the national or PCT international filing date of this application.

(Application Serial No.)	(Filing Date)	(Status)
		(patented. pending, abandoned)

Method for Improved Selectivity in Photo-Activation
and Detection of Molecular Diagnostic Agents
Declaration and Power of Attorney
PHO-0002   - Page 1 of 2 -

We hereby declare that all statements made herein of our own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under 18 U.S.C. § 1001 and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

We hereby appoint the following attorney, with full power of substitution and revocation. to prosecute this application and to transact all business in the United States Patent and Trademark Office connected therewith and request that all correspondence and telephone calls in respect to this application be directed to RICHARD M. KESSLER, ESQ. 11324 Gates Mill Drive, Knoxville, Tennessee 37922-1752. Telephone No. (423) 777-0244.

Richard M. Kessler   Registration No.: 35,214

Full name of first inventor ___ Eric A. Wachter _____

Inventor's signature _____ ___October 30. 1996___
                                                                     Date

Residence ___ 138 Bav Path Drive. Oak Ridge. Tennessee 37830 _____
Citizenship ___ United States of America _____
Post Office Address ___ 138 Bav Path Driv _____
___ Oak Ridge. Tennessee 37830 _____
                    * * *

Full name of second joint inventor ___ Walter G. Fisher _____

Inventor's signature _____ ___October 30. 1996___
                                                                     Date

Residence ___ 8514 Carl Valentine Road. Knoxville. Tennessee 37931 _____
Citizenship ___ United States of America _____
Post Office Address ___ 8514 Carl Valentine Road _____
___ Knoxville. Tennessee 37931 _____
                    * * *

Full name of third joint inventor ___ H. Craig Dees _____

Inventor's signature _____ ___October 30. 1996___
                                                                     Date

Residence ___ 1805 Sun Chase Apartments. 790 North Cedar Bluff Road. Knoxville. TN 37923
Citizenship ___ United States of America _____
Post Office Address ___ 1805 Sun Chase Apartments. 790 North Cedar Bluff Road __
___ Knoxville. TN 37923 _____
                    * * *

ADDRESS CORRESPONDENCE: RICHARD M. KESSLER, ESQ.
                                 11324 Gates Mill Drive
                                 Knoxville, Tennessee 37922-1752
                                 (423) 777-0244

Method for Improved Selectivity in Photo-Activation
and Detection of Molecular Diagnostic Agents
Declaration and Power of Attorney
PHO-0002   - Page 2 of 2 -

United States Patent & Trademark Office

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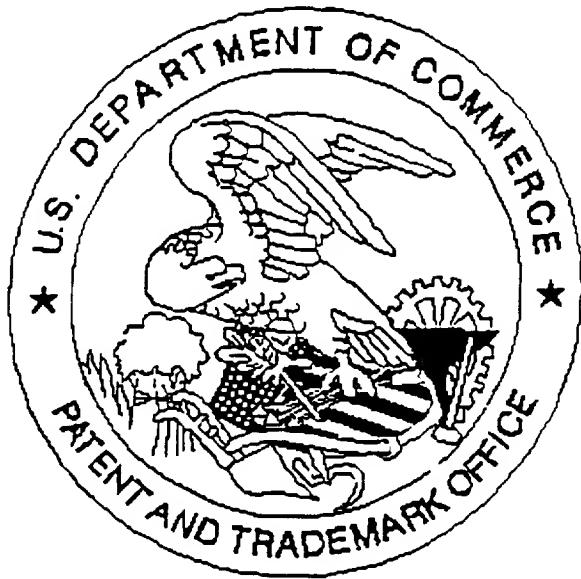
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